PATENT

Docket No. 12254/

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of

Inventor(s): Prof. Dr. Thomas Hunig

WARNING: Patent must be applied for in the name(s) of all of the actual inventor(s). 37 CFR 1.41(a) and 1.53(b).

For (title): Human CD28 Specific Monoclonal Antibodies for Antigen-Non-Specific Activation of T-Lymphocytes

1. Type of Application
This new application is for a(n) (check one applicable item below):

X Original

_ Design

_ Plant

NOTE: If one of the following 3 items apply then complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF A PRIOR U.S. APPLICATION CLAIMED and a NOTIFICATION IN PARENT APPLICATION OF THE FILING OF THIS CONTINUATION APPLICATION.

_ Divisional (37 CFR § 1.60)
_ Continuation
_ Continuation-in-part (CIP)

CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this New Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service on this date **11/24/99** in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number **EL335700298US** addressed to the: Assistant Commissioner for Patents, Box Patent Application, Washington, D.C. 20231.

Laura S. Kaplan
(Type or print name of person mailing paper)

Laura S. Kaplan
(Signature of person mailing paper)

2. Benefit of Prior U.S. Application(s) (35 USC 120)

NOTE: If the new application being transmitted is a divisional continuation or a continuation-in-part of a parent case, or where the parent case is an International Application which designated the U.S., then check the following item and complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

X The new application being transmitted claims the benefit of prior U.S. application(s) and enclosed are ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

3. Papers Enclosed Which Are Required For Filing Date Under 37 CFR 1.53(b) (Regular) or 37 CFR 1.153 (Design) Application

11 Pages of specification

3 Pages of claims

1 Pages of Abstract

14 Sheets of drawing

X formal
_ informal

NOTE: "Identifying indicia such as the serial number, group and unit, title of the invention, attorney's docket number, inventor's name, number of sheets, etc., not to exceed 2 3/4 inches (7.0 cm) in width may be placed in a centered location between the side edges within three fourths inch (19.1 mm) of the top edge. Either this marking technique on the front of the drawing or the placement, although not preferred, of this information and the title of the invention on the back of the drawings is acceptable." Proposed 37 CFR 1.84(1). Notice of March 9, 1988 (1090 O.G. 57-62).

4. Additional papers enclosed

X Preliminary Amendment

_ Information Disclosure Statement (37 CFR 1.98)

_ Form PTO-1449

_ Citations

_ Declaration of Biological Deposit

_ Submission of "Sequence Listing," computer readable copy and/or amendment pertaining thereto for biotechnology invention containing nucleotide and/or amino acid sequence.

_ Authorization of Attorney(s) to Accept and Follow Instructions from Representative

_ Special Comments

_ Other

5. Declaration or oath

_ Enclosed
executed by (check all applicable boxes)

_ inventor(s).

_ legal representative of inventor(s). 37 CFR 1.42 or 1.43

_ joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached.

_ this is the petition required by 37 CFR 1.47 and the statement required by 37 CFR 1.47 is also attached. See Item 13 below for fee.

X Not Enclosed. (Will follow)

_ Application is made by a person authorized under 37 CFR 1.41(c) on behalf of all the above named inventor(s). (The declaration or oath, along with the surcharge required by 37 CFR 1.16(e) can be filed subsequently).

NOTE: It is important that all the correct inventor(s) are named for filing under 37 CFR 1.41(c) and 1.53(b).

_ Showing that the filing is authorized. (Not required unless called into question. 37 CFR 1.41(d).

6. Inventorship Statement

WARNING: If the named inventors are each not the inventors of all the claims an explanation, including the ownership of the various claims at the time the **last** claimed invention was made, should be submitted.

The inventorship for all the claims in this application are:

X The same

or

_ Are not the same. An explanation, including the ownership of the various claims at the time the **last** claimed invention was made.

_ is submitted.
_ will be submitted.

7. Language

_ English

X non-English

X the attached translation is a verified translation. 37 CFR 1.52(d).

8. Assignment

_ An assignment of the invention to _____

_ is attached. A separate ____ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or _ FORM PTO 1906 is also attached.

_ will follow.

NOTE: "If an assignment is submitted with a new application, send two separate letters-one for the application and one for the assignment." Notice of May 4, 1990 (1114 O.G. 77-78).

9. Certified Copy

Certified copy(ies) of application(s)

PCT	PCT/DE98/01499	May 28, 1998
(country)	(appln. no.)	(filed)
(country)	(appln. no.)	(filed)
(country)	(appln. no.)	(filed)

from which priority is claimed

X is(are) attached.

_ will follow.

10. Fee Calculation (37 CFR 1.16)

A. X Regular application

CLAIMS AS FILED				
Number filed	**Number Extra**		**Rate**	**Basic Fee 37 CFR 1.16(a) $760.00**
Total Claims (37 CFR 1.16(c)) 40 - **20**=	20	X	**$18.00**/9.00	$360.00
Independent Claims (37 CFR 1.16(b)) 1 - **3**=	0	X	**$78.00**/39.00	$0.00
Multiple dependent claim(s), if any (37 CFR 1.16(d))	0		**$260.00**/130	$0.00

_ Amendment cancelling extra claims enclosed.

_ Amendment deleting multiple dependencies enclosed.

X Fee for extra claims is not being paid at this time.

NOTE: If the fees for extra claims are not paid on filing they must be paid or the claims cancelled by amendment, prior to the expiration of the time period set for response by the Patent and Trademark Office in any notice of fee deficiency. 37 CFR 1.16(d).

Filing Fee Calculation $1,120.00

B. _ **Design application**
($310.00--37 CFR 1.16(f))
Filing Fee Calculation $_____

C. _ **Plant application**
($480.00--37 CFR 1.16(9))
Filing fee calculation $_____

11. Small Entity Statement(s)

_ Verified Statement(s) that this is a filing by a small entity under 37 CFR 1.9 and 1.27 is(are) attached.
Filing Fee Calculation (50% of A, B or C above)
$_____

12. Request for International-Type Search (37 CFR 1.104(d)) (complete, if applicable)

_ Please prepare an international-type search report for this application at the time when national examination on the merits takes place.

13. Fee Payment Being Made At This Time

_ Not Enclosed

_ No filing fee is to be paid at this time. (This and the surcharge required by 37 CFR 1.16(e) can be paid subsequently.)

X Enclosed

X basic filing fee $ 760.00

_ recording assignment ($40.00; 37 CFR 1.21 (h)) $_____

_ petition fee for filing by other than all the inventors or person on behalf of the inventor where inventor refused to sign or cannot be reached. ($130.00; 37 CFR 1.47 and 1.17(h)) $_____

_ for processing an application with a specification in a non-English language. ($130.00; 37 CFR 1.52(d) and 1.17(k) $_____

_ processing and retention fee ($130.00; 37 CFR 1.53(d) and 1.21 (l)) $_____

_ fee for international-type search report ($40.00; 37 CFR 1.21 (e)). $_____

Total fees enclosed $ 760.00

14. Method of Payment of Fees

__ Check in the amount of $_____.

_x_ Charge Account No. 04-0932 in the amount of $760.00 . This transmittal is submitted in triplicate.

NOTE: Fees should be itemized in such a manner that it is clear for which purpose the fees are paid. 37 CFR 1.22(b).

15. Authorization to Charge Additional Fees

WARNING: If no fees are to be paid on filing the following items should not be completed.
WARNING: Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges, if extra claim charges are authorized.

_X_ The Commissioner is hereby authorized to charge the following additional fees by this paper and during the entire pendency of this application to Account No. 04-0932 :

__ 37 CFR 1.16(a), (f) or (g) (filing fees)

_X_ 37 CFR 1.16(b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 CFR 1.16(d)), it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.

_X_ 37 CFR 1.16(e) (surcharge for filing the basic filing fee and/or declaration on a date later than the filing date of the application)

_X_ 37 CFR 1.17 (application processing fees)

__ 37 CFR 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 CFR 1.311 (b))

16. Instructions As To Overpayment

_X_ credit Account No. 04-0932

__ refund

SIGNATURE OF ATTORNEY

Reg. No.: 37,221

Paul C. Remus
Type or print name of attorney

Tel. No. (603) 669-1000

Devine, Millimet & Branch, P.A.

P.O. Address

111 Amherst Street, P.O. Box 719
Manchester, New Hampshire 03105

_ **Incorporation by reference of added pages**

Check the following item if the application in this transmittal claims the benefit of prior U.S. application(s) (including an international application entering the U.S. stage as a continuation, divisional or C-I-P application) and complete and attach the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED

x Plus Added Pages For New Application Transmittal Where Benefit Of Prior U.S. Application(s) Claimed

Number of pages added 5

X Plus Added Pages For Papers Referred To In Item 4 Above

Number of pages added 8

_ Plus "Assignment Cover Letter Accompanying New Application"

Number of pages added ____

_ **Statement Where No Further Pages Added**

(If no further pages form a part of this Transmittal then end this Transmittal with this page and check the following item)

X This transmittal ends with this page.

Docket No. 11254/

ADDED PAGES FOR APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED

NOTE: "In order for an application to claim the benefit of a prior filed copending national application, the prior application must name as an inventor at least one inventor named in the later filed application and disclose the named inventor's invention claimed in at least one claim of the later filed application in the manner provided by the first paragraph of 35 U.S.C. 112." 37 CFR 1.78(a).

NOTE: "In addition the prior application must be (l) complete as set forth in § 1.51, or (2) entitled to a filing date as set forth in § 1.53(b) and include the basic filing fee set forth in § 1.16; or (3) entitled to a filing date as set forth in § 1.53(b) and have paid therein the processing and retention fee set forth in § 1.21(1) within the time period set forth in § 1.53(d)." 37 CFR 1.78(a).

17. Relate Back

NOTE: "Any application claiming the benefit of a prior filed copending national or international application must contain or be amended to contain in the first sentence of the specification following the title a reference to such prior application identifying it by serial number and filing date or international application number and international filing date and indicating the relationship of the applications." 37 CFR 1.78(a). See also the Notice of April 28, 1987 (1079 O.G. 32 to 46).

[X] Amend the Specification by inserting before the first line the sentence:

This patent application claims priority to PCT Application Number PCT/DE98/01499 with an international filing date of May 28, 1998.

A. 35 U.S.C. 119(e)

NOTE: Any nonprovisional application claiming the benefit of one or more prior filed copending provisional applications must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior provisional application, identifying it as a provisional application, and including the provisional application number (consisting of series code and serial number)." 37 C.F.R. § 1.78(a)(4).

[] "This application claims the benefit of U.S. Provisional Application(s) No(s).:

APPLICATION NO(S).:	FILING DATE
_____/_____ "	_____
_____/_____ "	_____
_____/_____ "	_____

B. 35 U.S.C. 120, 121 and 365(c)

NOTE: "Any nonprovisional application claiming the benefit of one or more prior filed copending nonprovisional applications or international applications designating the United States of America must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior application, identifying it by application number (consisting of the series code and serial number) or international application number and international filing date and indicating the relationship of the applications. Cross-references to other related applications may be made when appropriate. (See § 1.14(b))." 37 C.F.R. § 1.78(2).

[] "This application is a

[] continuation
[] continuation-in-part
[] divisional

of copending application(s)

[] application number 0_/_____ filed on _____"

[] International Application _____ filed on _____ and which designated the U.S."

NOTE: The proper reference to a prior filed PCT application which entered the U.S. national phase is the U.S. serial number and the filing date of the PCT application which designated the U.S.

NOTE: (1) Where the application being transmitted adds subject matter to the International Application then the filing can be as a continuation-in-part or (2) if it is desired to do so for other reasons, e.g. where no declaration is available, no English translation is available or no fee is to be paid on filing then the filing can be as a continuation. In these cases the International Application designating the U.S. is treated as the parent case in the U.S. and is an alternative to the completion of the International Application under 35 U.S.C. 371(c)(4) which must meet the requirements of 37 CFR 1.61(a). This alternative permits the completion of the filing requirements within any term set by the PTO under 37 CFR 1.53(d) to which the extension provisions of 37 CFR 1.136(a) apply. (Whereas, if the filing is as an international application entering the U.S. stage then the fee, declaration and/or English translation (where necessary) is due within 20 months of the priority date but can be paid within 22 months of the priority date (or is due within 30 months of the priority date but can be submitted within 32 months of the priority date) with the surcharges set forth in 37 CFR 1.492(e), (f) and 37 CFR 1.495(c); however, the provisions of 37 CFR 1.136 do not apply to this 22 or (32 month) period. 37 CFR 1.61(b).)

[] "The nonprovisional application designated above, namely application ____/_____, filed _____, claims the benefit of U.S. Provisional Application(s) No(s).:

APPLICATION NO(S).:	**FILING DATE**
_____/_____	_____"
_____/_____	_____"
_____/_____	_____"

NOTE: The deadline for entering the national phase in the U.S. for an international application was clarified in the Notice of April 28, 1987 (1079 O.G. 32 to 46) as follows:

"The Patent and Trademark Office considers the International application to be pending until the 22nd month from the priority date if the United States has been designated and no Demand for International Preliminary Examination has been filed prior to the expiration of the 19th month from the priority date and until the 32nd month from the priority date if a Demand for International Preliminary Examination which elected the United States of America has been filed prior to the expiration of the 19th month from the priority date, provided that a copy of the international application has been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively. If a copy of the international application has not been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively, the international application becomes abandoned as to the United States 20 or 30 months from the priority date respectively. These periods have been placed in the rules as paragraph (h) of § 1.494 and paragraph (i) of § 1.495. A continuing application under 35 U.S.C. 365(c) and 120 may be filed anytime during the pendency of the international application. "

18. Relate Back--35 U.S.C. 119 Priority Claim for Prior Application

The prior U.S. application(s), including any prior International Application designating the U.S., identified above in item 17, in turn itself claim(s) foreign priority (ies) as follows:

PCT	PCT/DE98/01499	May 28, 1998
country	appln. no.	filed on

The certified copy (ies) has (have)

[] been filed on _____ in prior application 0_/_____ , which was filed on _____ —.

[X] is (are) attached

WARNING: The certified copy of the priority application which may have been communicated to the PTO by the International Bureau may **not** be relied on without any need to file a certified copy of the priority application **in the continuing application**. This is so because the certified copy of the priority application communicated by the International Bureau is placed in a folder and is not assigned a U.S. serial number unless the national stage is entered. Such folders are disposed of if the national stage is not entered. Therefore such certified copies may not be available if needed later in the prosecution of a continuing application. An alternative would be to physically remove the priority documents from the folders and transfer them to the continuing application. The resources required to request transfer, retrieve the folders, make suitable record notations, transfer the certified copies, enter and make a record of such copies in the Continuing Application are substantial. Accordingly, the priority documents in folders of international applications which have not entered the national stage may not be relied on. Notice of April 28, 1987 (1079 O.G. 32 to 46).

19. Maintenance of Copendency of Prior Application

NOTE: The PTO finds it useful if a copy of the petition filed in the prior application extending the term for response is filed with the papers constituting the filing of the continuation application. Notice of November 5, 1985(1060 O.G. 27).

A. [] Extension of time in prior application

*(This item **must** be completed and the papers filed **in the prior application** if the period set in the prior application has run)*

[] A petition, fee and response extends the term in the pending prior application until _____

[] A copy of the petition filed in prior application is attached

B. [] Conditional Petition for Extension of Time in Prior Application

(complete this item if previous item not applicable)

[] A conditional petition for extension of time is being filed in the pending prior application.

[] A copy of the conditional petition filed in the prior application is attached

20. Further Inventorship Statement Where Benefit of Prior Application(s) Claimed

NOTE: "If the continuation, continuation-in-part, or divisional application is filed by less than all the inventors named in the prior application a statement **must** accompany the application when filed requesting deletion of the names of the person or persons who are not inventors of the invention being claimed in the continuation, continuation-in-part, or divisional application. " 37 CFR 1.62(a) [emphasis added]. (dealing with the file wrapper continuation situation).

NOTE: "In the case of a continuation-in-part application which adds and claims additional disclosure by amendment, an oath or declaration as required by § 1.63 must be filed. In those situations where a new oath or declaration is required due to additional subject matter being claimed, additional inventors may be named in the continuing application. In a continuation or divisional application which discloses and claims only subject matter disclosed in a prior application, no additional oath or declaration is required and the application must name as inventors the same or less than all the inventors in the prior application." 37 CFR 1.60(c). (dealing with the continuation situation).

(complete applicable item (a), (b) and/or (c) below)

(a) [X] This application discloses and claims only subject matter disclosed in the prior application whose particulars are set out above and the inventor(s) in this application are

[X] the same

[] less than those named in the prior application and it is requested that the following inventor(s) identified for the prior application be deleted:

_____

(Type name(s) of inventor(s) to be deleted)

(b) [] This application discloses and claims additional disclosure by amendment and a new declaration or oath is being filed. With respect to the prior application the inventor(s) in this application are

[] the same

[] the following additional inventor(s) have been added

_____

(Type name(s) of inventor(s) to be added)

(c) The inventorship for all the claims in this application are

[X] the same

[] not the same, and an explanation, including the ownership of the various claims at the time the last claimed invention was made

[] is submitted
[] will be submitted

21. Abandonment of Prior Application (if applicable)

[] Please abandon the prior application at a time while the prior application is pending or when the petition for extension of time or to revive in that application is granted and when this application is granted a filing date so as to make this application copending with said prior application.

NOTE: According to the Notice of May 13, 1983 (103, TMOG 6-7) the filing of a continuation or continuation-in-part application is a proper response with respect to a petition for extension of time or a petition to revive and should include the express abandonment of the prior application conditioned upon the granting of the petition and the granting of a filing date to the continuing application.

22. Petition for Suspension of Prosecution for the Time Necessary to File an Amendment

WARNING: "The claims of a new application may be finally rejected in the first Office action in those situations where (1) the new application is a continuing application of, or a substitute for, an earlier application, and (2) all the claims of the new application (a) are drawn to the same invention claimed in the earlier application, and (b) would have been properly finally rejected on the grounds of art of record in the next Office action if they had been entered in the earlier application." MPEP, § 706.07(b).

NOTE: Where it is possible that the claims on file will give rise to a first action final for this continuation application and for some reason an amendment cannot be filed promptly (e.g., experimental data is being gathered) it may be desirable to file a petition for suspension of prosecution for the time necessary.

(check the next item, if applicable)

[] There is provided herewith a Petition To Suspend Prosecution for the Time Necessary to File An Amendment (New Application Filed Concurrently)

23. Small Entity (37 CFR § 1.28(a))

[] Applicant has established small entity status by the filing of a verified statement in parent application _____/_____ on _____.

[] A copy of the verified statement previously filed is included.

WARNING: "Status as a small entity in one application or patent does not affect any other application or patent, including applications or patents which are directly or indirectly dependent upon the application or patent in which the status has been established. Applications filed as continuations, divisions or continuations-in-part of a parent application must include a reference to a verified statement filed in the parent application if status as a small entity is still proper and desired." 37 CFR § 1.28(a).

24. NOTIFICATION IN PARENT APPLICATION OF THIS FILING

[] A notification of the filing of this

(check one of the following)

[] continuation

[] continuation-in-part

[] divisional

is being filed in the parent application from which this application claims priority under 35 USC § 120.

Jungblut
Prelim. Amd.U.S. Nat. Phase
PCT/DE98/01499
Nov. 22, 1999

Attorney Docket No. 11254/

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: Hunig, T.

Group Art Unit: Not assigned

Serial No.: PCT/DE 98/01499

Examiner: Unknown

Filed (International Priority Date): 5/28/97

Docket No.: 11254/

For: Human CD28 Specific Monoclonal Antibodies for Antigen-Non-Specific Activation of T-Lymphocytes

Paper No.: 1

To: Assistant Commissioner for Patents
Box PCT
Washington, D.C. 20231

Dear Sir:

CERTIFICATE OF MAILING (37 C.F.R. 1.10)

In hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the United States Postal Service on the date shown below with sufficient postage as Express Mail: No: EL335700298US _____ in an envelope post office to addressee: Assistant Commissioner for Patents, Box PCT, Washington, D.C. 20231

Date: 11/24/99

Laura S. Kapka

PRELIMINARY AMENDMENT

Please amend the above identified application for entry into the U.S. National Phase under 35 U.S.C. section 371 as follows.

In The Claims:

Please Amend Following Claims:

In claim 3, line 1, please delete "claims 1 or 2," and substitute therefor: -- claim 1 -- .

In claim 4, line 2, please delete "one of the claims 1 to 3" and substitute therefor: -- claim 1 --.

In claim 5, lines 1 and 2, please delete "one of the claims 1 to 3" and substitute therefor: -- claim 1 --.

In claim 7, line 1, please delete "one of the claims 1 to 3" and substitute therefor: -- claim 1 --.

In claim 11, line 1, please delete "one of the claims 1 to 3" and substitute therefor: -- claim 1 --.

In claim 12, line 1, please delete "one of the claims 1 to 3" and substitute therefor: -- claim 1 --.

Please Add the Following New Claims:

13. Monoclonal antibodies according to claim 2, with the hybridoma cells enabled to produce monoclonal human-CD28 specific animal antibodies being available through

a) creation of a plasmid by means of insertion of human-CD28 cDNA into the pHβAPr-1-neo vector following excision of the Sall-Hindlll fragment and production of protoplasts from Escherichia coli (MC 1061) with carry the plasmid,

b) fusing the protoplasts with mouse A20J and/or L929 tumor cells by means of polyethylene glycol,

c) cultivation of the transfected cells received in phase b),

d) screening of the transfected mouse A20J and/or L929 cells for the expression of human CD28 and selection of mouse A20J and/or L929 cells expressing human-CD28,

e) immunization of BALB/c mice with mouse A20J and or L929 cells expressing human-CD28,

f) removal of spleen cells of the mice immunized in this way and fusing the spleen cells with cells of the cell line X63-Ag 8.653 by means of polyethylene glycol,

g) selection of the hybridoma cells received in this way with the condition that in the supernatant of selected hybridoma cells there are antibodies contained which bind on human CD28 expressing mouse A20J and/or L929 cells and

h) cultivation/sub-cloning of the selected hybridoma cells obtained in phage g).

14. Hybridoma cells for the production of monoclonal antibodies according to claim 2 which are available through the following procedural steps:

a) creation of a plasmid by means of insertion of human-CD28 cDNA into the pHβAPr-1-neo vector following excision of the Sall-Hindlll fragment and production of protoplasts from Escherichia coli (MC 1061) with carry the plasmid,

b) fusing the protoplasts with mouse A20J and/or L929 tumor cells by means of polyethylene glycol,

c) cultivation of the transfected cells received in phase b),

d) screening of the transfected mouse A20J and/or L929 cells for the expression of human CD28 and selection of mouse A20J and/or L929 cells expressing human-CD28,

e) immunization of BALB/c mice with mouse A20J and or L929 cells expressing human-CD28,

f) removal of spleen cells of the mice immunized in this way and fusing the spleen cells with cells of the cell line X63-Ag 8.653 by means of polyethylene glycol,

g) selection of the hybridoma cells received in this way with the condition that in the supernatant of selected hybridoma cells there are antibodies contained which bind on human CD28 expressing mouse A20J and/or L929 cells.

15. Hybridoma cells for the production of monoclonal antibodies according to claim 3 which are available through the following procedural steps:

a) creation of a plasmid by means of insertion of human-CD28 cDNA into the pHβAPr-1-neo vector following excision of the Sall-Hindlll fragment and production of protoplasts from Escherichia coli (MC 1061) with carry the plasmid,

b) fusing the protoplasts with mouse A20J and/or L929 tumor cells by means of polyethylene glycol,

c) cultivation of the transfected cells received in phase b),

d) screening of the transfected mouse A20J and/or L929 cells for the expression of human CD28 and selection of mouse A20J and/or L929 cells expressing human-CD28,

e) immunization of BALB/c mice with mouse A20J and or L929 cells expressing human-CD28,

f) removal of spleen cells of the mice immunized in this way and fusing the spleen cells with cells of the cell line X63-Ag 8.653 by means of polyethylene glycol,

g) selection of the hybridoma cells received in this way with the condition that in the supernatant of selected hybridoma cells there are antibodies contained which bind on human CD28 expressing mouse A20J and/or L929 cells.

16. Procedure for the production of monoclonal antibodies according to claim 2 with the following procedural steps:

a) production of hybridoma cells enabled to produce monoclonal human-CD28 specific animal antibodies by means of an immunization with non-T tumor cell lines on which human-CD28 is expressed,

b) if applicable, humanization of the monoclonal animal antibodies available from the hybridoma cells pursuant to phase a) through a biochemical or gene-technological exchange of constant components of the animal antibodies against analogous constant components of a human antibody or replacement of genes or the hybridoma cells corresponding to the components;

c) secreting of the antibody in hybridoma cell cultures and isolation of the antibodies from it or production of the antibodies by injection of the hybridoma cells into animals, for example, mice, and isolation of the antibodies from the body fluid of the animal.

17. Procedure for the production of monoclonal antibodies according to claim 3 with the following procedural steps:

a) production of hybridoma cells enabled to produce monoclonal human-CD28 specific animal antibodies by means of an immunization with non-T tumor cell lines on which human-CD28 is expressed,

b) if applicable, humanization of the monoclonal animal antibodies available from the hybridoma cells pursuant to phase a) through a biochemical or gene-technological exchange of constant components of the animal antibodies against analogous constant components of a human antibody or replacement of genes or the hybridoma cells corresponding to the components;

c) secreting of the antibody in hybridoma cell cultures and isolation of the antibodies from it or production of the antibodies by injection of the hybridoma cells into animals, for example, mice, and isolation of the antibodies from the body fluid of the animal.

18. Use of monoclonal antibodies according to claim 2 for the production of a medicine for the therapeutic treatment of the human body.

19. Use of monoclonal antibodies according to claim 3 for the production of a medicine for the therapeutic treatment of the human body.

20. Use of monoclonal antibodies according to claim 2 for the treatment of diseases of the human body.

21. Use of monoclonal antibodies according to claim 3 for the treatment of diseases of the human body.

22. Procedures for the therapeutic treatment of the human body with monoclonal antibodies according to claim 2 being used.

23. Procedures for the therapeutic treatment of the human body with monoclonal antibodies according to claim 3 being used.

24. Hybridoma cells for the production of monoclonal antibodies according to claim 13 which are available through the following procedural steps:

a) creation of a plasmid by means of insertion of human-CD28 cDNA into the pHβAPr-1-neo vector following excision of the Sall-HindIII fragment and production of protoplasts from Escherichia coli (MC 1061) with carry the plasmid,

b) fusing the protoplasts with mouse A20J and/or L929 tumor cells by means of polyethylene glycol,

c) cultivation of the transfected cells received in phase b),

d) screening of the transfected mouse A20J and/or L929 cells for the expression of human CD28 and selection of mouse A20J and/or L929 cells expressing human-CD28,

e) immunization of BALB/c mice with mouse A20J and or L929 cells expressing human-CD28,

f) removal of spleen cells of the mice immunized in this way and fusing the spleen cells with cells of the cell line X63-Ag 8.653 by means of polyethylene glycol,

g) selection of the hybridoma cells received in this way with the condition that in the supernatant of selected hybridoma cells there are antibodies contained which bind on human CD28 expressing mouse A20J and/or L929 cells.

25. Procedure for the production of monoclonal antibodies according to claim 13 with the following procedural steps:

a) production of hybridoma cells enabled to produce monoclonal human-CD28 specific animal antibodies by means of an immunization with non-T tumor cell lines on which human-CD28 is expressed,

b) if applicable, humanization of the monoclonal animal antibodies available from the hybridoma cells pursuant to phase a) through a biochemical or gene-technological exchange of constant components of the animal antibodies against analogous constant components of a human antibody or replacement of genes or the hybridoma cells corresponding to the components;

c) secreting of the antibody in hybridoma cell cultures and isolation of the antibodies from it or production of the antibodies by injection of the hybridoma cells into animals, for example, mice, and isolation of the antibodies from the body fluid of the animal.

26. Use of monoclonal antibodies according to claim 13 for the production of a medicine for the therapeutic treatment of the human body.

27. Use of monoclonal antibodies according to claim 13 for the treatment of diseases of the human body.

28. Procedures for the therapeutic treatment of the human body with monoclonal antibodies according to claim 13 being used.

29. Procedure according to claim 16, with the hybridoma cells enabled to produce monoclonal human-CD28 specific animal antibodies being produced in the following procedural steps:

a) creation of a plasmid by means of insertion of human-CD28 cDNA into the pHβAPr-1-neo vector following excision of the Sall-Hindlll fragment and production of protoplasts from Escherichia coli (MC1061) which carry the plasmid,

b) fusing of the protoplasts with mouse A20J and/or L929 tumor cells by means of polyethylene glycol,

c) cultivation of the transfected cells received in phase b),

d) screening of the transfected mouse A20J and /or L929 cells for the expression of human-CD28 and selection of mouse A20J and/or L929 cells expressing human-CD28,

e) immunization of BALB/c mice with mouse A20J and/or L929 cells expressing human-CD28,

f) removal of spleen cells of the mice immunized in this way and fusing the spleen cells with cells of the cell line X63-Ag 8.653 by means of polyethylene glycol and

g) selection of the hybridoma cells received in this way with the condition that in the supernatant of selected hybridoma cells there are antibodies contained which bind on human-CD28 expressing mouse A20J and/or L929 cells.

30. Procedure according to claim 17, with the hybridoma cells enabled to produce monoclonal human-CD28 specific animal antibodies being produced in the following procedural steps:

a) creation of a plasmid by means of insertion of human-CD28 cDNA into the pHβAPr-1-neo vector following excision of the Sall-HindIII fragment and production of protoplasts from Escherichia coli (MC1061) which carry the plasmid,

b) fusing of the protoplasts with mouse A20J and/or L929 tumor cells by means of polyethylene glycol,

c) cultivation of the transfected cells received in phase b),

d) screening of the transfected mouse A20J and /or L929 cells for the expression of human-CD28 and selection of mouse A20J and/or L929 cells expressing human-CD28,

e) immunization of BALB/c mice with mouse A20J and/or L929 cells expressing human-CD28,

f) removal of spleen cells of the mice immunized in this way and fusing the spleen cells with cells of the cell line X63-Ag 8.653 by means of polyethylene glycol and

g) selection of the hybridoma cells received in this way with the condition that in the supernatant of selected hybridoma cells there are antibodies contained which bind on human-CD28 expressing mouse A20J and/or L929 cells.

31. Procedure according to claim 25, with the hybridoma cells enabled to produce monoclonal human-CD28 specific animal antibodies being produced in the following procedural steps:

a) creation of a plasmid by means of insertion of human-CD28 cDNA into the pHβAPr-1-neo vector following excision of the Sall-HindIII fragment and production of protoplasts from Escherichia coli (MC1061) which carry the plasmid,

b) fusing of the protoplasts with mouse A20J and/or L929 tumor cells by means of polyethylene glycol,

c) cultivation of the transfected cells received in phase b),

d) screening of the transfected mouse A20J and /or L929 cells for the expression of human-CD28 and selection of mouse A20J and/or L929 cells expressing human-CD28,

e) immunization of BALB/c mice with mouse A20J and/or L929 cells expressing human-CD28,

f) removal of spleen cells of the mice immunized in this way and fusing the spleen cells with cells of the cell line X63-Ag 8.653 by means of polyethylene glycol and

g) selection of the hybridoma cells received in this way with the condition that in the supernatant of selected hybridoma cells there are antibodies contained which bind on human-CD28 expressing mouse A20J and/or L929 cells.

32. Use according to claim 18 for the production of a medicine for the treatment of diseases with pathologically reduced numbers of CD4 T cells, in particular AIDS or following stem cell transplantations after chemotherapy of leukemic diseases.

33. Use according to claim 19 for the production of a medicine for the treatment of diseases with pathologically reduced numbers of CD4 T cells, in particular AIDS or following stem cell transplantations after chemotherapy of leukemic diseases.

34. Use according to claim 26 for the production of a medicine for the treatment of diseases with pathologically reduced numbers of CD4 T cells, in particular AIDS or following stem cell transplantations after chemotherapy of leukemic diseases.

35. Use according to claim 18 for the production of a medicine for the potentiation and/or qualitative influencing of immune reactions in protective inoculations.

36. Use according to claim 19 for the production of a medicine for the potentiation and/or qualitative influencing of immune reactions in protective inoculations.

37. Use according to claim 26 for the production of a medicine for the potentiation and/or qualitative influencing of immune reactions in protective inoculations.

38. Use according to claim 18 for the production of a medicine to influence the quality of the T cell reaction; in particular to influence the production of various effector molecules, for example cytokines and chemokines and their receptors, for example in auto-immune diseases and AIDS.

39. Use according to claim 19 for the production of a medicine to influence the quality of the T cell reaction; in particular to influence the production of various effector molecules, for example cytokines and chemokines and their receptors, for example in auto-immune diseases and AIDS.

40. Use according to claim 26 for the production of a medicine to influence the quality of the T cell reaction; in particular to influence the production of various effector molecules, for example cytokines and chemokines and their receptors, for example in auto-immune diseases and AIDS.

Jungblut
Prelim. Amd.U.S. Nat. Phase
PCT/DE98/01499
Nov. 22, 1999

Remarks:

Applicant submits this preliminary amendment to become part of the disclosure of the application submitted herewith to enter the U.S. National Phase of PCT Application No. PCT/DE98/01499.

Applicant has amended the claims simply to remove the multiple dependent claims present in the International Application. No new matter has been added. Applicant respectfully contends that, based upon the above remarks and amendments, Applicant submits that the application is now in condition for allowance and early notice of such allowance is respectfully requested. The fees associated with this amendment, if any are due, are included herewith.

Respectfully submitted,

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Human CD28 specific monoclonal antibodies for antigen-non-specific activation of T-lymphocytes

Description:

The invention in question is concerned with monoclonal antibodies which are specific for human CD28 and activate T-lymphocytes without occupancy of an antigen receptor of the T-lymphocytes, i.e. antigen-non-specific, hybridoma cells for the production of such antibodies, a procedure for the production of such antibodies and applications of such antibodies. Monoclonal antibodies is the term for antibodies which are produced by hybrid cell lines (so-called hybridoma), which have resulted from a fusion of a B cell of animal or human origin producing antibodies with a suitable myelom tumour cell. CD28 designates a cell surface module of a known amino-acid sequence expressed on T-lymphocytes of human and animal origin, to which the abbreviation CD28 was given in the course of the international "Human Leukocyte Typing Workshops". With the activation of T-lymphocytes, an increase in metabolic activity, enlargement of the cell volume, synthesis of immunologically important molecules and the start of cell division (proliferation) of T-lymphocytes upon an external stimulus is meant. For example, these processes are initiated by the occupancy of the CD28 molecule on T cells by certain CD28-specific monoclonal antibodies. The activation of T-lymphocytes with the peripheral phenomena described is a part of the physiological immune reaction, but can get out of control there (lymphoproliferative diseases) or be insufficient (immune deficiency) in pathological situations.

To start with, the following technological background is necessary in order to understand the invention. The activation of resting T cells for proliferation and functional differentiation firstly demands the occupancy of two surface structures, so-called receptors: 1. the antigen receptor, which possesses a differing specificity from cell to cell and is necessary for the recognition of antigens, e.g. viral fission products; and the CD28 molecule expressed on all resting T cells to the same extent, which naturally binds on ligands on the surface of other cells of the immune system. We talk of the "co-stimulation" of the antigen-specific immune reaction through CD28. In cell cultures, these processes can be imitated by occupying the antigen receptor and the CD28 molecule with suitable monoclonal antibodies. In the classical system of co-stimulation, neither the occupancy of the antigen receptor nor that of the CD28 molecule alone leads to T cell proliferation, whereas the occupancy of both receptors is effective. This observation was made on T cells in man, mice and rats.

Monoclonal antibodies of the kind stated at the beginning are known. A "direct" activation of resting T-lymphocytes by CD28-specific monoclonal antibodies, i.e. activation independent of the occupancy of the antigen receptor, has been observed in the following systems: in the literature Brinkmann et al., J. Immunology, 1996, 156, 4100-4106, it was shown that a very small fraction (5%) of human T-lymphocytes bearing the surface marker CD45 R0, which is typical for

resting T-lymphocytes, is activated by the "classical" CD28-specific monoclonal antibody 9.3 with addition of the growth factor Interleukin-2 (IL-2) without the occupancy of the antigen receptor. In the work by Siefken et al., Cellular Immunology, 1997, 176: 59-65, it was shown that a CD28-specific monoclonal antibody produced by conventional means, i.e. by immunisation of mice with human T cells, can activate a sub-group of human T cells in cell culture without occupancy of the antigen receptor for proliferation if CD28 is occupied by this monoclonal antibody and the cell-bound monoclonal antibody molecules are additionally crosslinked with one another by further antibodies. In both cases, the antibodies described are firstly not principally suited for use in human medicine, as they are mouse antibodies. Further, the two described antibodies have the common point that only a very small part of the T cells can be activated "directly".

In the work by Tacke et al., Eur. J. Immunol., 1997, 27:239-247, two kinds of CD28-specific monoclonal antibodies with varying functional properties were described: "classical antibodies", which only co-stimulate the activation of resting T cells with the simultaneous occupancy of the antigen receptor; and "direct" ones, which can activate T-lymphocytes of all classes in vitro and in animals to proliferation without occupancy of the antigen receptor. Both monoclonal antibodies known in this regard come from an immunisation with cells on which rat CD28 is expressed and are available by various selections aimed at the properties described for each of them. Further, this literature shows that CD28-specific monoclonal antibodies possessing the directly activating effect bind on T-lymphocytes much more slowly than classical CD28-specific monoclonal antibodies; the binding on a mouse fibroblast cell line (L-929), on the surface of which the CD28 molecule is artificially expressed by transfection, takes place for classical and "directly" stimulating CD28-specific monoclonal antibodies at the same speed. From this, it is deduced that the "directly" stimulating CD28-specific monoclonal antibodies known in this regard recognise an active form of the CD28 molecule, the existence on which on resting T cells is suppressed by a mechanism as yet unknown, but which is accessible in expression of the molecule in non-T tumour cell lines. However, the monoclonal antibodies known in this regard are on the one hand specific against rat CD28, on the other hand mouse antibodies. They are therefore not suitable for therapeutic purposes in man for both reasons.

Compared with the state of the art as per the first two pieces of literature stated, the invention in question is based on the technical problem of making "direct" human-CD28 specific monoclonal antibodies available which on the one hand are human-compatible and on the other hand are able to activate human T cells to a large extent.

As a solution to this technical problem, the invention in question teaches human-compatible monoclonal antibodies which are specific for human CD28 and human T-lymphocytes of several to all sub-groups to activate without occupancy of an antigen receptor of the human T-lymphocytes and thus antigen-non-

specifically, preferably with human constant components. - Constant components of an antibody are areas which are not of importance for the recognition of an antigen, as opposed to the variable areas, which define the antigen-specificity of an antibody. Constant components however differ in antibodies of various kinds and consequently also animals and humans. The constant areas of an antibody must correspond to those of antibodies of an organism which is to be treated with the antibodies in order to be compatible. Monoclonal antibodies in accordance with the invention are therefore human-compatible on the one hand, be it per se or by humanisation, and can on the other hand be used to treat various diseases which are based on an excessively low T-lymphocyte activity, as the antibodies are specific against human CD28 and as the activation of the T-lymphocytes is extensive.

The invention in question naturally includes various derivatives of monoclonal antibodies provided the features stated in the claims are fulfilled. Derivatives of monoclonal antibodies means modifications of the monoclonal antibodies which are generated by customary biochemical or gene-technical manipulations. This exists, for example, in the humanisation of monoclonal antibodies of a mouse through partial substitution of structural (constant) components of the mouse antibody through those of a human one.

In detail, monoclonal antibodies according to the invention are available through: A) production of hybridoma cells enabled to produce monoclonal human-CD28 specific animal antibodies by means of an immunisation with non-T tumour cell lines on which human CD28 is expressed, B) if applicable, humanisation of the monoclonal animal antibodies available from the hybridoma cells pursuant to phase A through a biochemical or gene-technological exchange of constant components of the animal antibodies against analogous constant components of a human antibody or replacement of genes of the hybridoma cells corresponding to the components; C) secreting of the antibodies in hybridoma cell cultures and isolation of the antibodies from it or production of the antibodies by injection of the hybridoma cells into animals, for example mice, and isolation of the antibodies from the body fluid of the animal. Accordingly, compared with the nearest places in literature, Brinkmann et al., J. Immunology, 1996, 156, 4100-4106, and Siefken et al., Cellular Immunology, 1997, 176: 59-65, the core of the invention entails the knowledge that a "direct" activation of practically all the T-lymphocytes can be achieved if the monoclonal antibodies are obtained by immunisation with non-T tumour cells on which human CD28 is expressed instead of an immunisation with T cell lines. In this way, monoclonal antibodies can be obtained which are not only specific against human CD28, but also cause a "direct" activation to a considerable extent. In detail, monoclonal antibodies according to the invention have specificity for determinants of the human CD28 molecule, which are difficult to access on the naturally expressed CD28 molecule and the occupancy of which by the new kind of monoclonal antibodies leads to an activation of the T cells. A determinant means the area of a molecule which is defined by the binding specificity of one or more antibodies.

The principal mode of procedure in the production of hybridoma cells, in humanisation and in the production of monoclonal antibodies from (humanised) hybridoma cells is well known to experts and need not be explained in any more detail here. As a matter of principle, all the customary, known and freely available cell lines, in particular for the production of the hybridoma cells, can be used here. For the production of the monoclonal antibodies, not only the mode of procedure described below, but also recombinant expression, which is known in detail to experts, is to be considered.

In detail, it is preferable if the hybridoma cells enabled to produce monoclonal human-CD28 specific animal antibodies are available through a) creation of a plasmid by means of insertion of human-CD28 cDNA into the pHβAPr-1-neo vector following excision of the SalI-HindIII fragment and production of protoplasts from Escherichia coli (MC1061) which carry the plasmid, b) fusing of the protoplasts with mouse A20J and/or L929 tumour cells by means of polyethylene glycol, c) cultivation of the transfected cells received in phase b, d) screening of the transfected mouse A20J and/or L929 cells for the expression of human CD28 and selection of mouse A20J and/or L929 cells expressing human-CD28, e) immunisation of BALB/c mice with mouse A20J and/or L929 cells expressing human-CD28 (for example by injection 6 x i.p. and then 1 x i.v.), f) removal of spleen cells of the mice immunised in this way and fusing the spleen cells with non-producer cells of the cell line X63-Ag 8.653 (i.e. not producing any antibodies) by means of polyethylene glycol, g) selection of the hybridoma cells obtained in this way with the condition that in the supernatant of selected hybridoma cells there are antibodies contained which bind on human CD28 expressing mouse A20J and/or L929 cells and h) cultivation/sub-cloning of the selected hybridoma cells obtained in phase g. Naturally, other expression systems known to the experts could be used instead of phases a) to d). Human-CD28 cDNA is freely available from Dr. A. Aruffo and Dr. B. Seed, who published the sequence and also the following part of literature: Aruffo, A., and Seed, B., 1987, "Molecular cloning of a CD28 cDNA by a high efficiency COS cell expression system", Proc. Natl. Acad. Sci. USA, 84:8573. Therefore, the production of the human CD28 cDNA can be seen in detail from this literature. In addition, each expert can very simply and quickly produce a human-CD28 cDNA clone with the help of the sequence deposited in the gene bank and the polymerase chain reaction. The pHβAPr-1-neo vector is freely available from the authors of the literature Gunning, P., et al., 1987, "A human β-actin expression vector system directs high-level accumulation of antisense transcripts", Proc. Natl. Acad. Sci. USA, 84:4831. In this, "neo" stands for neomycin resistance. Phase c) is therefore carried out with the presence of neomycin. The cell lines and/or micro-organisms mentioned above are freely available and can be purchased from the American Type Culture Collection (ATCC). With regard to Escherichia coli (MC1061), supplementary reference is made to the literature Meissner, P.S., et al., 1987, "Bacteriophage gamma cloning system for the construction of directional cDNA libraries", Proc. Natl. Acad. Sci. USA, 84:4171.

Accordingly, the object of the invention also includes, pursuant to patent claim 4, hybridoma cells as well as a procedure for the production of antibodies according to the invention pursuant to patent claims 5 and 6.

However, within the framework of the invention, the use of monoclonal antibodies according to the invention for the production of medicines, in particular the treatment of diseases with pathologically reduced CD4-T cell figures, such as AIDS or in stem cell transplantations following chemotherapy of leukemic diseases, for potentiating and/or qualitative influence of immune reactions in protective inoculations and/or in order to influence the quality of T cell reactions, in particular to influence the production of various effector molecules, for example cytokines and chemokines and their receptors, e.g. in auto-immune diseases and AIDS, is of independent importance. The galenic production of the medicines for the various forms of administration is well known to experts and need not be explained in more detail here. The quality of the T cell reaction is in particular to be understood as the production of certain cytokine patterns, which can, for example, be effective in a pro or anti-inflammatory way or can selectively lead to the production of certain classes of immunglobulin in B lymphocytes (classic examples of differing qualities of the T cell reaction are the function TH1 and TH2 phenotypes, as described below in examples). The invention also entails procedures for the healing of the diseases mentioned both above and below, making use of monoclonal antibodies according to the invention.

Below, the invention is explained in more detail on the basis of examples of implementation. In particular, the production of monoclonal antibodies according to the invention is described. In these examples, screening procedures with which monoclonal antibodies according to the invention and hybridoma cells used as a basis can be selected also become clear in detail. Therapeutic possibilities of use according to the invention also become clear in the following examples.

The experiments portrayed and the examples of the effects of "direct" CD28-specific monoclonal antibodies were carried out in the animal model of rats, with the monoclonal antibody JJ319 being used as an example of a "classical" CD28-specific antibody, the monoclonal antibody JJ316 being used as an example of a "directly" activating one. Both antibodies are freely available and can be purchased from the firm of Pharmingen, San Diego, USA. JJ319 and JJ316 antibodies are also available in accordance with literature, M. Tack et al., Immunology, 1995, 154: 5121-5127, to which reference is hereby expressly made, also with regard to details of the production of hybridoma cells and monoclonal antibodies.

Example 1

In this example, the production of monoclonal antibodies according to the invention, i.e. human CD28-specific, is described. They are also termed below as CMY-2. Human CD28 from a cDNA library was recombinantly expressed in

A20J and/or L929 cell lines. To start with, a plasmid was created by means of insertion of human-DC28 cDNA into the pHβAPr-1-neo vector following excision of the SalI-HindIII fragment. Protoplasts, which carry the plasmid, were produced from Escherichia coli (MC1061). There was then a fusing of the protoplasts with mouse A20J and/or L929 tumour cells by means of polyethylene glycol. The transfected cells obtained in this way were cultivated in the customary way. There was then a screening of the transfected mouse A20J and/or L929 cells for expression of human-CD28 and selection of mouse A20J and/or L929 cells expressing human-CD28.

Proof of the successful expression was done with the help of a conventional, commercially available fluorescence-marked antibody with specificity for human CD28 (9.3-phycoerythrin). As a negative check, non-transfected A20J or L929 cells were stained with the same antibody. The transfectants (A20J-CD28 and L929-CD28) showed a higher fluorescence intensity. As not all the cells were CD28 positive, CD28 positive cells were sub-cloned and used for immunisation. As can be seen in Fig. 1 from the displacement of the clouds of dots to the top in the two right-hand diagrams, these cells reacted with the purchased antibody, i.e. expressed human CD28 on their surface.

The A20J human-CD28 cell line was used for the immunisation of BALB/C mice. Cell fusion and screening were carried out as follows: i) immunisation of BALB/c mice with the mouse A20J cells expressing the human-CD28 (injections 6 x i.p. and then 1 x i.v.). ii) Removal of spleen cells of the mice immunised in this way and fusing of the spleen cells with cells of the X63-Ag 8.653 cell line by means of polyethylene glycol. iii) Selection of the hybridoma cells obtained in this way with the condition that in the supernatant of selected hybridoma cells there are antibodies contained which bind on mouse A20J and/or L929 cells expressing human-CD28.

The read-out was the staining of a mixture of CD28 transfected and untransfected L929 tumour cells. Fig. 2 shows that the CMY-2 monoclonal antibody isolated in this way distinguishes transfected and untransfected cells by differences in the fluorescence intensity. The differential screening for antibodies against human-CD28 was done as follows. 50 µl each of supernatant of cultivated cell hybridoma were removed and incubated for 15 min with a mixture of L929 cells and L929-CD28 transfectants. After washing, the cells were stained with DaMIg-PE. Part A shows the negative check. The cells were only incubated with DaMIg-PE. Part B shows the coloration with a supernatant which was slightly positive, but shows no difference in the two cells. Part C shows the cells stained with a supernatant of CMY-2.

In experiments which have not been portrayed, peripheral blood cells of man were stained with the newly isolated CMY-2 and the "classical", CD28-specific antibody 9.1. An identical expression pattern was found on the sub-populations of human blood cells.

To sum up, the experiments show that CMY-2 is a human CD28-specific antibody.

CMY-2 was then tested with human T-lymphocytes from peripheral blood enriched to about 80% for classical co-stimulating and for "directly" stimulating activity. The T cell proliferation was measured by adding ^{3}H-thymidin between the 2nd and 3rd day of the culture. The following results were achieved:

Co-stimulation:	
Unstimulated cells	276 cpm
CD3-specific antibodies	3111 cpm
CD3-specific antibodies + CMY-2	51676 cpm
Direct stimulation:	
Solid-phase anti-mouse Ig	379 cpm
Solid-phase anti-mouse Ig + control mAk	258 cpm
Solid-phase anti-mouse Ig plus CMY-2	19115 cpm

As an explanation: Anti-CD3 ensures T cell receptor stimulation (CD3 is a part of the TCR complex). CMY-2 was used in the form of an uncleaned culture supernatant (50% final volume). Experience has shown that the effective mAk concentration to be expected is sub-optimal for a direct activation, but sufficient for the co-stimulation. The experiment shows that CMY-2 has directly activating properties.

Hybridoma cells according to the invention which produce CMY-2 have been deposited with the DMSZ, the German Collection of Micro-organisms and Cell Cultures, Mascheroder Weg 1b, D-38124 Braunschweig, under number DSM ACC2353 (20.05.98).

Example 2

In this example, the immune-modulating effect of "direct" CD28-specific monoclonal antibodies is explained in more detail. The objective of the experiments is proof of the lead cytokines of the TH2 cells, IL-4 and IL-10, as a result of the direct activation in vitro and in vivo. IL-4 is decisive for the cooperation of the TH2 cells with B lymphocytes, for the differentiation of further CD4 T cells to TH2 cells and thus the polarisation of the immune system away from the inflammation and towards the humoral immune reaction, and IL-10 is the central factor for the suppression of inflammatory (TH1) reactions. Only the more important results of the in vivo treatment of rats have been portrayed. In vitro, even clearer effects were found.

Fig. 3 shows the expressed cytokine profile of lymphatic nodes and spleen cells of young LEW rats three days after i.p. injection of the directly activating mAk JJ316, the co-stimulator JJ319, the TCR-specific mAk R73 or the vehicle PBS. The figure shows a so-called RNAse protection test, in which radioactively

marked antisense mRNA samples are protected against the decomposition of added RNAse. These go onto bands defined on the gel and make it possible to have a good overview of the expressed cytokine profile on the mRNA level of a tissue at a glance. The two smallest fragments, L32 and GADPH, are "household genes", the identical expression of which is used for the check of equally large amounts of RNA used in the individual cases. The test was carried out with a kit commercially available from the firm of Pharmingen.

JJ316, not JJ319 or R73, massively induce IL-10 and, to a lesser extent, IL-4 mRNA. The effects are especially clear in the spleen, but are also visible in lymph nodes.

In Fig. 4, the cytokine IL-4 on a protein and simultaneously on a single-cell level is proven by flow-cytophotometric analysis. For this purpose, the cells are firstly stained with mAk against the surface molecule CD4, then fixed and made permeable, with the result that the IL-4 protein can be proven in a subsequent cytoplasmatic staining with an IL-4-specific mAk which is marked with a second fluoride chrome. The evaluation is done in a flow-cytophotometer, each point representing a cell. The quadrants inserted represent the borders between the background and a positive reaction. The methods are also described in great detail in the catalogue of the firm of Pharmingen.

As Fig. 4 shows, the injection of the mAk JJ316, but not that of the classical co-stimulator JJ319, induces the production of IL-4 in a substantial share of the isolated CD4 T cells.

In Fig. 5, a biological effect of the increased IL-4 production is shown: the level of detectable antibodies of the IGE class clearly rises as a result of the treatment with mAk JJ316 and proves the in vivo effectivity of the IL-4 secretion induced by "direct" CD28-specific monoclonal antibodies.

Figures 6 and 7 show so-called EMSAs (Electrophoretic Mobility Shift Assays) as a proof of the induction of transcription factors which support the development of anti-inflammatory TH2 cells. The technique is as follows. T cells are stimulated in vitro for differing lengths of time, then the proteins from the cell cores are put into solution and incubated with a radioactively marked short gene probe, the sequence of which they should recognise as transcription factors. After the incubation, the mixture is separated on a poly-acrylamide gel. The unbound marked gene probe exudes from the gel at the bottom (cannot be seen here). Bands such as those portrayed here are partly non-specific (exist everywhere) or are selectively induced (strong, weak signals, depending upon the stimulus).

The following becomes clear from looking at Figures 6 and 7. Both with a GATA3-specific as well as with a c-Maf-response-element-specific sample, increased induction in co-stimulation (TCR and classical CD28) as well as direct stimulation ("direct" CD28) can be seen in comparison with the stimulation only

via the T cell receptor (TCR). To sum up, it can be stated that stimulation of the CD28 molecule supports the expression of these transcription factors in the cell core and that this is also possible without TCR stimulation by direct CD28 stimulation. Reference is made to Current Opinion in Immunology 1997, 9:776-781, for the importance of these factors for the differentiation of TH2 cells.

The interconnections for the immune-regulatory and immune-modulating effect of "direct" CD28-specific monoclonal antibodies with regard to the formation of TH1 and/or TH2 cells explained above in detail consequently make them especially suited for the production of medicine for the treatment of such immune reactions of independent diseases. Principally, these are all the allergic-inflammatory and auto-immune-inflammatory clinical pictures. The former include, for example, the inflammatory diseases of the intestines summarised under the designation "Inflammatory Bowel Disease" (IBD) and contact dermatitis. The latter includes Type I diabetes and multiple sclerosis. It can also be expected that a strong stimulation of the human CD28 molecule by monoclonal antibodies according to the invention will be in a position to cure T cells infected by HIV I. In this way, chemokine receptors used by the viruses as cellular co-receptors can be eliminated an the production of chemokines, which bind on such receptors and thus block them for HIV I viruses, can be induced.

Example 3

Fig. 8 shows the proliferative reaction of unseparated lymphatic node cells of rats to the "directly" stimulating CD28-specific monoclonal antibody (JJ316) and the lack of such a reply in the use of a "classical" CD28-specific monoclonal antibody (JJ319). The cells were cultivated for 2 days in 0.2 ml of medium (RPMI 1640, available for GIBCO/BRL, containing 5% FCS [fetal calf serum]) in presence or absence of the above mentioned additives with a density of 1 million cells per ml in a gassed incubator. The cell division activity was determined by the addition of radioactively marked thymidin (1µCi/insertion for 16 hours, 1Ci - 37GBq, determination with β detector).

Unlike published results (Siefken et al., Cellular Immunology, 1997, 176: 59-65), this result shows that, for the T cell activation by directly activating CD28-specific monoclonal antibodies, it is not necessary to crosslink them artificially by means of a second antibody. Rather, the presence of non-T cells from lymphoid organs, viz. from B lymphocytes and so-called accessory cells, is sufficient in order to make a direct activation by solubly added CD28-specific monoclonal antibodies possible. This probably happens through the binding of the monoclonal antibodies to so-called Fc receptors of these non-T cells. This result is an important precondition for the therapeutic use of "directly" stimulating CD28-specific monoclonal antibodies, in which an artificial crosslinking with anti-immunglobulin antibodies in the entire organism is not practicable.

Example 4

"Directly" activating CD28-specific monoclonal antibodies lead to an increase in the number of CD4 T cells in an intact organism. Fig. 9 shows this for lymphatic nodes in rats which had been given 1 mg of the "directly" stimulating CD28-specific monoclonal antibody (JJ316) or of the "classical" CD28-specific monoclonal antibody (JJ319) on day 0. With directly activating monoclonal antibodies according to the invention with a specificity for human-CD28 and their ability to stimulate the proliferation of T-lymphocytes, totally analogous effects were achieved. This can then be particularly applied in situations in which the share of CD4 T cells has been pathologically reduced and is to be brought back up towards the normal level again. Such situations can particularly be found in the clinical picture of AIDS and after chemotherapy and bone marrow transplantations. In this example, the number of CD4 T cells is only temporarily increased; this is due to the fact that healthy animals were treated with normal CD4 T cells. The "surplus" cells resulting from the proliferation stimulation are reduced by homeostatic mechanisms.

Example 5

As can be expected from the deductions made above from Figures 5-7, directly activating CD28-specific monoclonal antibodies can be used therapeutically, inter alia to prevent inflammatory auto-immune reactions. Fig. 10 shows an experiment on this for the so-called Adjuvans Arthritis in rats, a model system for certain forms of rheumatoid arthritis in humans. "Paw volume increase" shows the increase in the volume of the paw. "Healthy" data points show figures for healthy animals. As an isotype control, a monoclonal antibody of the same immunoglobin class with specificity for an irrelevant human cell surface molecule was used. AA stands for Adjuvans Arthritis. PBS stands for "Phosphate-buffered saline". W3/25 stands for a monoclonal antibody with specificity for the CD4 molecule of rats. The Adjuvans Arthritis is mediated by so-called TH1 cells. TH1 cells result from resting CD4 T cells in the course of the activation under the influence of certain soluble factors of the immune system, so-called cytokines. The counterparts of the TH1 cells are the TH2 cells, which have an anti-inflammatory effect and the induction of which is controlled by other cytokines. In the experiment shown in Figs. 10 and 11, the induction of the Adjuvans Arthritis was almost completely suppressed by the "directly" activating CD28-specific monoclonal antibody JJ316, measured on the joint swelling (Fig. 10) and the arthritic index (Fig. 11) after immunisation with mycobacteria in Adjuvans. The "classical" CD28-specific monoclonal antibody (JJ319) had the opposite effect, i.e. it deteriorated the clinical picture. From this, it can be seen, also for use in humans, that the immune reaction can be influenced by the application of conventional or "directly" stimulating CD28-specific monoclonal antibodies according to the invention, here in the sense of an "immune deviation" to TH1 or TH2. In other words, monoclonal antibodies according to the invention, but also "classical" monoclonal antibodies which are specific for human-CD28 (and/or are available by immunisation with T cells) cause an immune modulation. Such a purpose of application of "classical" monoclonal antibodies is also not known.

Therefore, the invention also affects the use of monoclonal antibodies specific against human-CD28 (available according to the above mentioned principal modes of procedure in immunisation with T cell lines or non-T cell lines expressing human-CD28) for the production of medicines for the modulation of immune reactions, viz. immune suppression (for example with human-CD28 analogues to JJ319) or immune reinforcement (for example with human-CD28 analogues to JJ316 such as CMY-2).

Patent claims:

1) Human-compatible monoclonal antibodies which are specific for human-CD28 and activate human T-lymphocytes of several to all sub-groups without occupancy of an antigen receptor of the human T-lymphocytes and thus antigen-non-specifically.

2) Monoclonal antibodies according to claim 1 which are available through
   A) production of hybridoma cells enabled to produce monoclonal human-CD28 specific animal antibodies by means of an immunisation with non-T tumour cell lines on which human CD28 is expressed,
   B) if applicable, humanisation of the monoclonal animal antibodies available from the hybridoma cells pursuant to phase A through a biochemical or gene-technological exchange of constant components of the animal antibodies against analogous constant components of a human antibody or replacement of genes of the hybridoma cells corresponding to the components;
   C) secreting of the monoclonal antibodies in hybridoma cell cultures and isolation of the monoclonal antibodies from it or production of the monoclonal antibodies by injection of the hybridoma cells into animals, for example mice, and isolation of the monoclonal antibodies from the body fluid of the animals.

3) Monoclonal antibodies according to claims 1 or 2, with the hybridoma cells enabled to produce monoclonal human-CD28 specific animal antibodies being available through
   a) creation of a plasmid by means of insertion of human-CD28 cDNA into the pHβAPr-1-neo vector following excision of the SalI-HindIII fragment and production of protoplasts from Escherichia coli (MC1061) which carry the plasmid,
   b) fusing of the protoplasts with mouse A20J and/or L929 tumour cells by means of polyethylene glycol,
   c) cultivation of the transfected cells received in phase b,
   d) screening of the transfected mouse A20J and/or L929 cells for the expression of human CD28 and selection of mouse A20J and/or L929 cells expressing human-CD28,
   e) immunisation of BALB/c mice with mouse A20J and/or L929 cells expressing human-CD28,
   f) removal of spleen cells of the mice immunised in this way and fusing the spleen cells with cells of the cell line X63-Ag 8.653 by means of polyethylene glycol,
   g) selection of the hybridoma cells received in this way with the condition that in the supernatant of selected hybridoma cells there are antibodies contained which bind on human CD28 expressing mouse A20J and/or L929 cells and
   h) cultivation/sub-cloning of the selected hybridoma cells obtained in phase g.

4) Hybridoma cells for the production of monoclonal antibodies according to one of the claims 1 to 3 which are available through the following procedural steps:
   a) creation of a plasmid by means of insertion of human-CD28 cDNA into the pHβAPr-1-neo vector following excision of the SalI-HindIII fragment and production of protoplasts from Escherichia coli (MC1061) which carry the plasmid,
   b) fusing of the protoplasts with mouse A20J and/or L929 tumour cells by means of polyethylene glycol,
   c) cultivation of the transfected cells received in phase b,
   d) screening of the transfected mouse A20J and/or L929 cells for the expression of human CD28 and selection of mouse A20J and/or L929 cells expressing human-CD28,
   e) immunisation of BALB/c mice with mouse A20J and/or L929 cells expressing human-CD28,
   f) removal of spleen cells of the mice immunised in this way and fusing the spleen cells with cells of the cell line X63-Ag 8.653 by means of polyethylene glycol and
   g) selection of the hybridoma cells received in this way with the condition that in the supernatant of selected hybridoma cells there are antibodies contained which bind on human CD28 expressing mouse A20J and/or L929 cells.

5) Procedure for the production of monoclonal antibodies according to one of the claims 1 to 3 with the following procedural steps:

   A) production of hybridoma cells enabled to produce monoclonal human-CD28 specific animal antibodies by means of an immunisation with non-T tumour cell lines on which human CD28 is expressed,
   B) if applicable, humanisation of the monoclonal animal antibodies available from the hybridoma cells pursuant to phase A through a biochemical or gene-technological exchange of constant components of the animal antibodies against analogous constant components of a human antibody or replacement of genes of the hybridoma cells corresponding to the components;
   C) secreting of the antibody in hybridoma cell cultures and isolation of the antibodies from it or production of the antibodies by injection of the hybridoma cells into animals, for example mice, and isolation of the antibodies from the body fluid of the animal.

6) Procedure according to claim 5, with the hybridoma cells enabled to produce monoclonal human-CD28 specific animal antibodies being produced in the following procedural steps:
   a) creation of a plasmid by means of insertion of human-CD28 cDNA into the pHβAPr-1-neo vector following excision of the SalI-HindIII

fragment and production of protoplasts from Escherichia coli (MC1061) which carry the plasmid,

b) fusing of the protoplasts with mouse A20J and/or L929 tumour cells by means of polyethylene glycol,

c) cultivation of the transfected cells received in phase b,

d) screening of the transfected mouse A20J and/or L929 cells for the expression of human CD28 and selection of mouse A20J and/or L929 cells expressing human-CD28,

e) immunisation of BALB/c mice with mouse A20J and/or L929 cells expressing human-CD28,

f) removal of spleen cells of the mice immunised in this way and fusing the spleen cells with cells of the cell line X63-Ag 8.653 by means of polyethylene glycol and

g) selection of the hybridoma cells received in this way with the condition that in the supernatant of selected hybridoma cells there are antibodies contained which bind on human CD28 expressing mouse A20J and/or L929 cells.

7) Use of monoclonal antibodies according to one of the claims 1 to 3 for the production of a medicine for the therapeutic treatment of the human body.

8) Use according to claim 7 for the production of a medicine for the treatment of diseases with pathologically reduced numbers of CD4 T cells, in particular AIDS or following stem cell transplantations after chemotherapy of leukemic diseases.

9) Use according to claim 7 for the production of a medicine for the potentiation and/or qualitative influencing of immune reactions in protective inoculations.

10) Use according to claim 7 for the production of a medicine to influence the quality of the T cell reaction; in particular to influence the production of various effector molecules, for example cytokines and chemokines and their receptors, for example in auto-immune diseases and AIDS.

11) Use of monoclonal antibodies according to one of the claims 1 to 3 for the treatment of diseases of the human body.

12) Procedures for the therapeutic treatment of the human body with monoclonal antibodies according to one of the claims 1 to 3 being used.

Abstract

The invention teaches human-compatible monoclonal antibodies which are specific against human CD28 and human T-lymphocytes of several to all sub-groups to activate without occupancy of an antigen receptor of the human T-lymphocytes and thus antigen-non-specifically.

I certify that the above translation from the German language is correct.

Grassel, 13 FEB 1999

KEITH J. LESTER, B.A.
sworn interpreter and translator for the English language for the courts and notaries of the District Court Circuit Braunschweig
GRASSEL

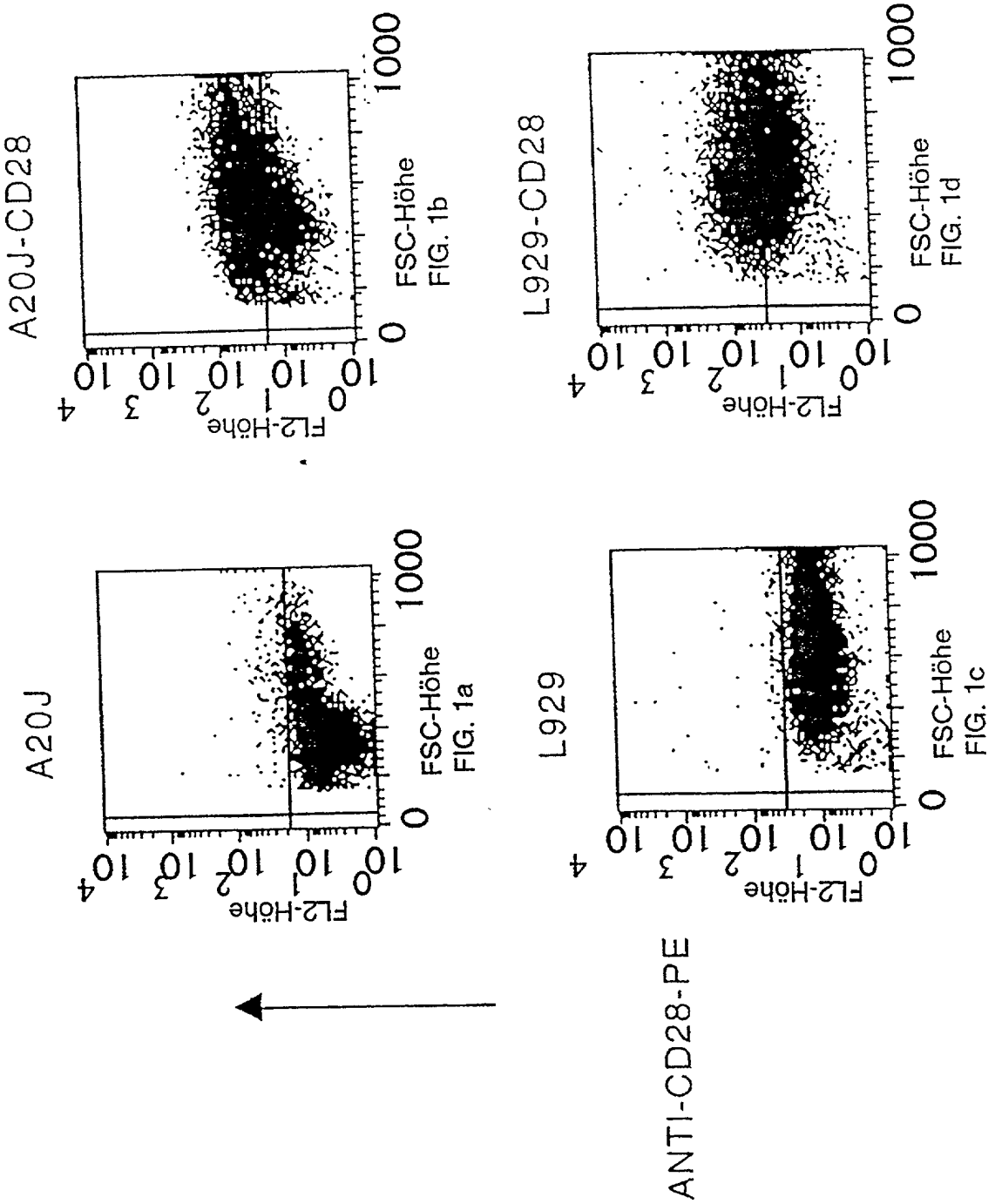


FIG. 2A

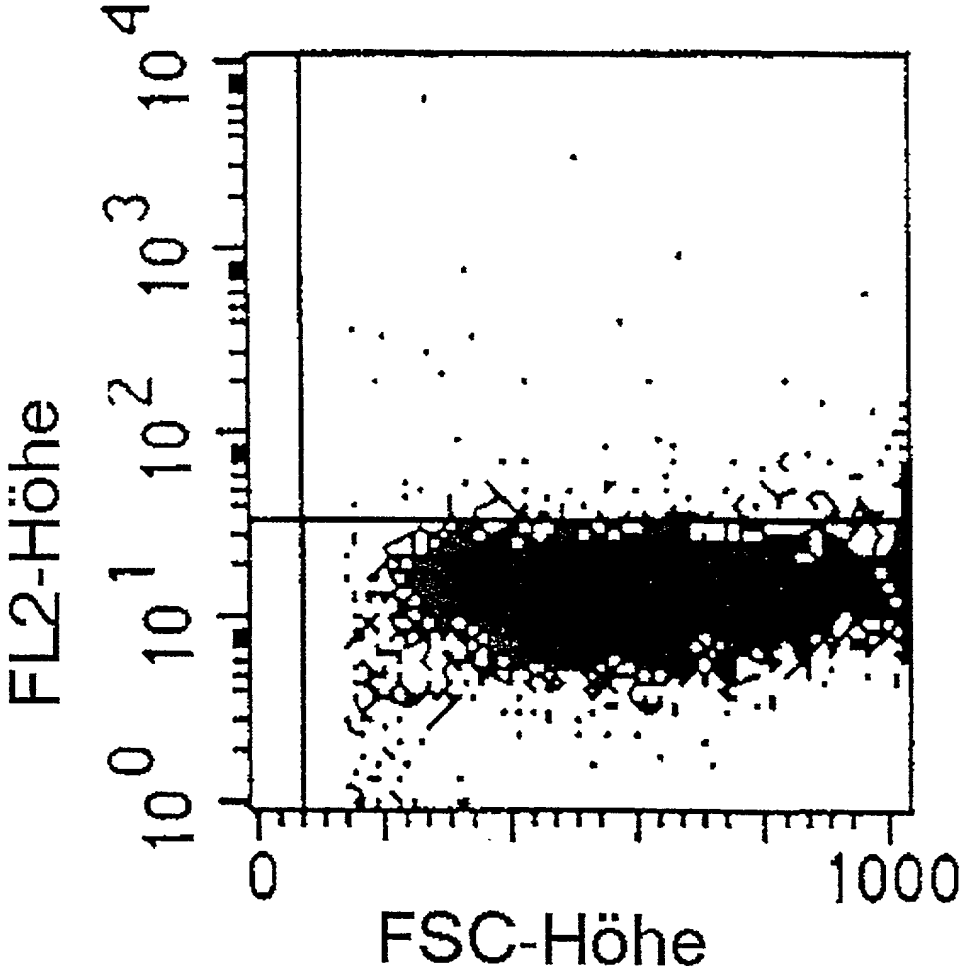


FIG. 2B

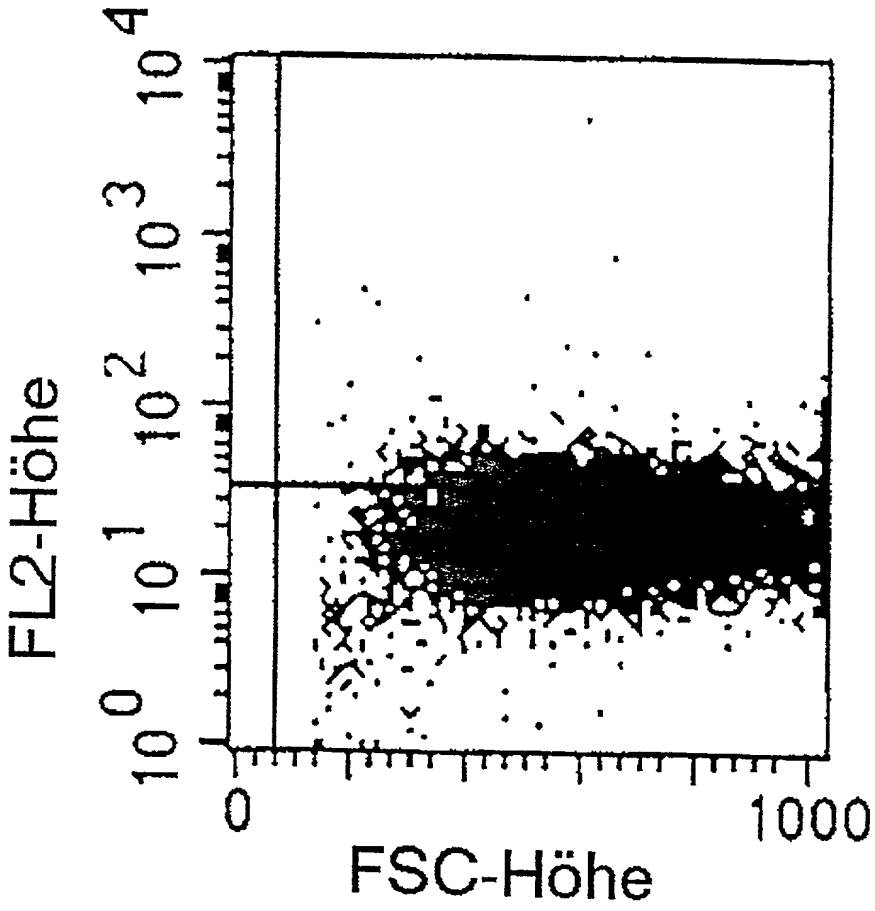


FIG. 2C

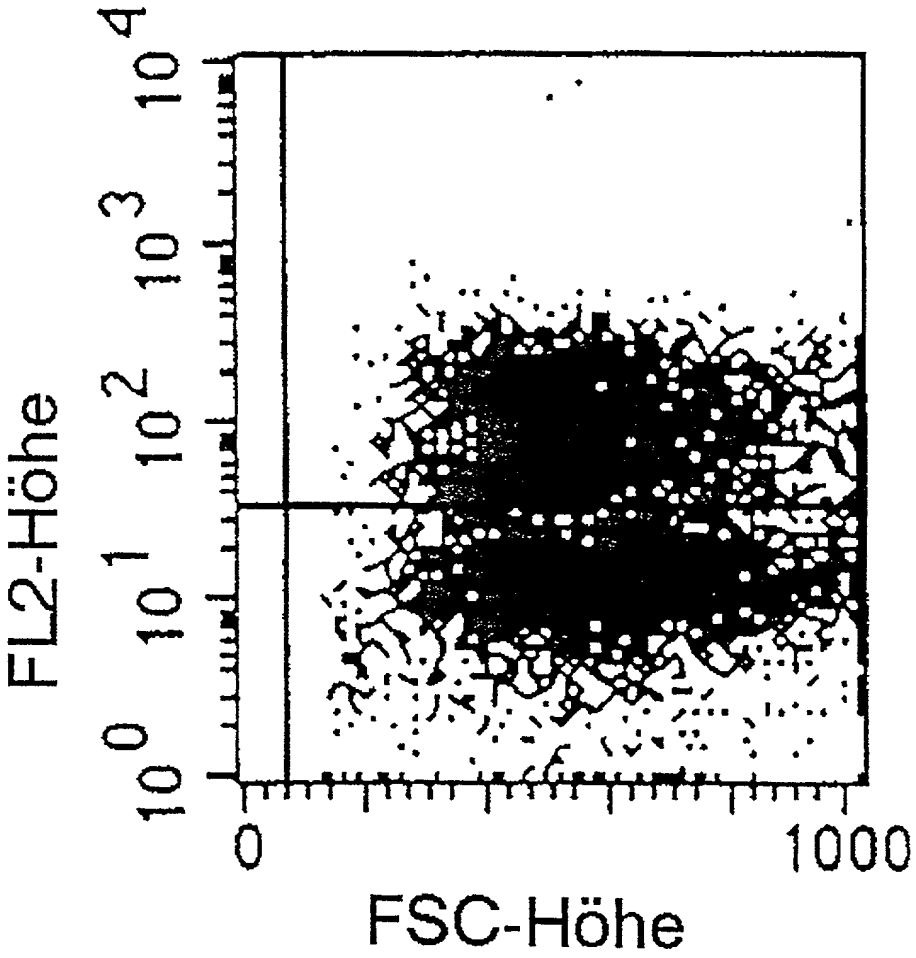


FIG. 3

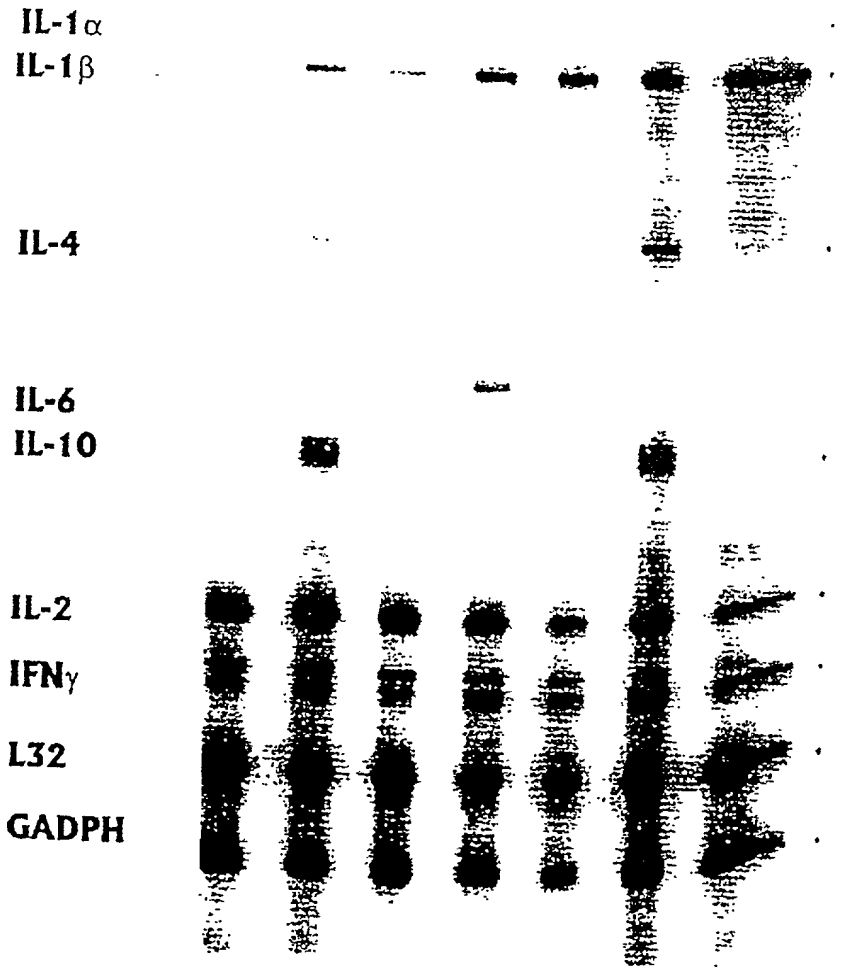


Fig. 4a

JJ319

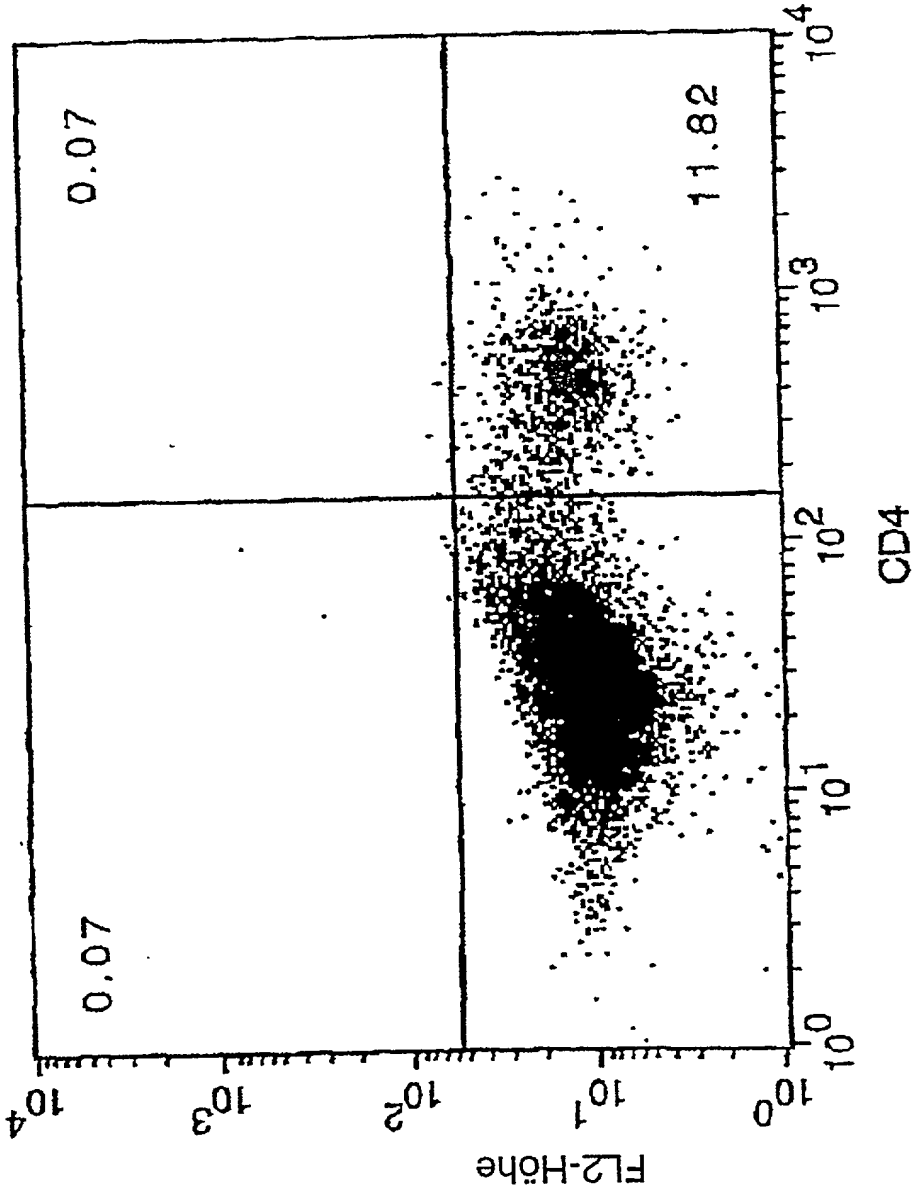
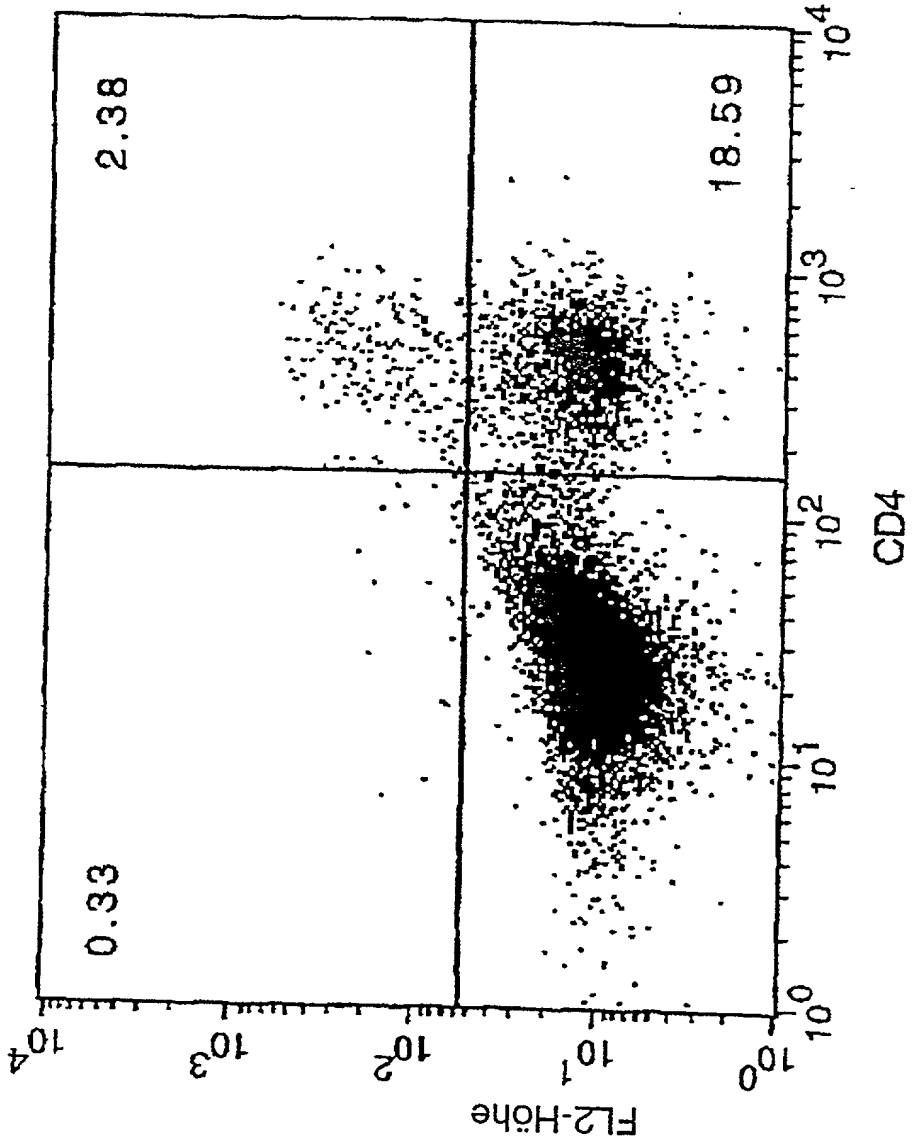


Fig. 4b

JJ316



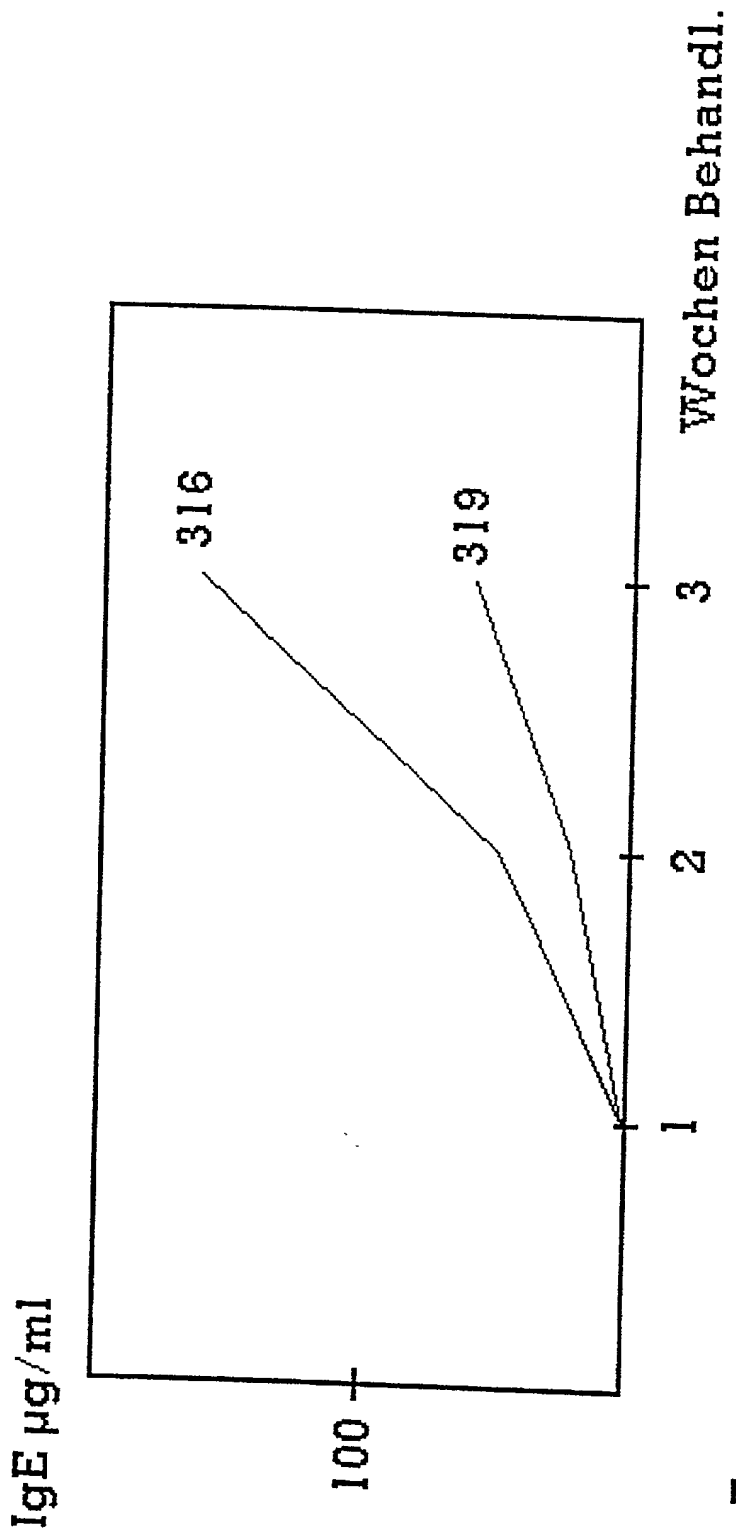


FIG. 5

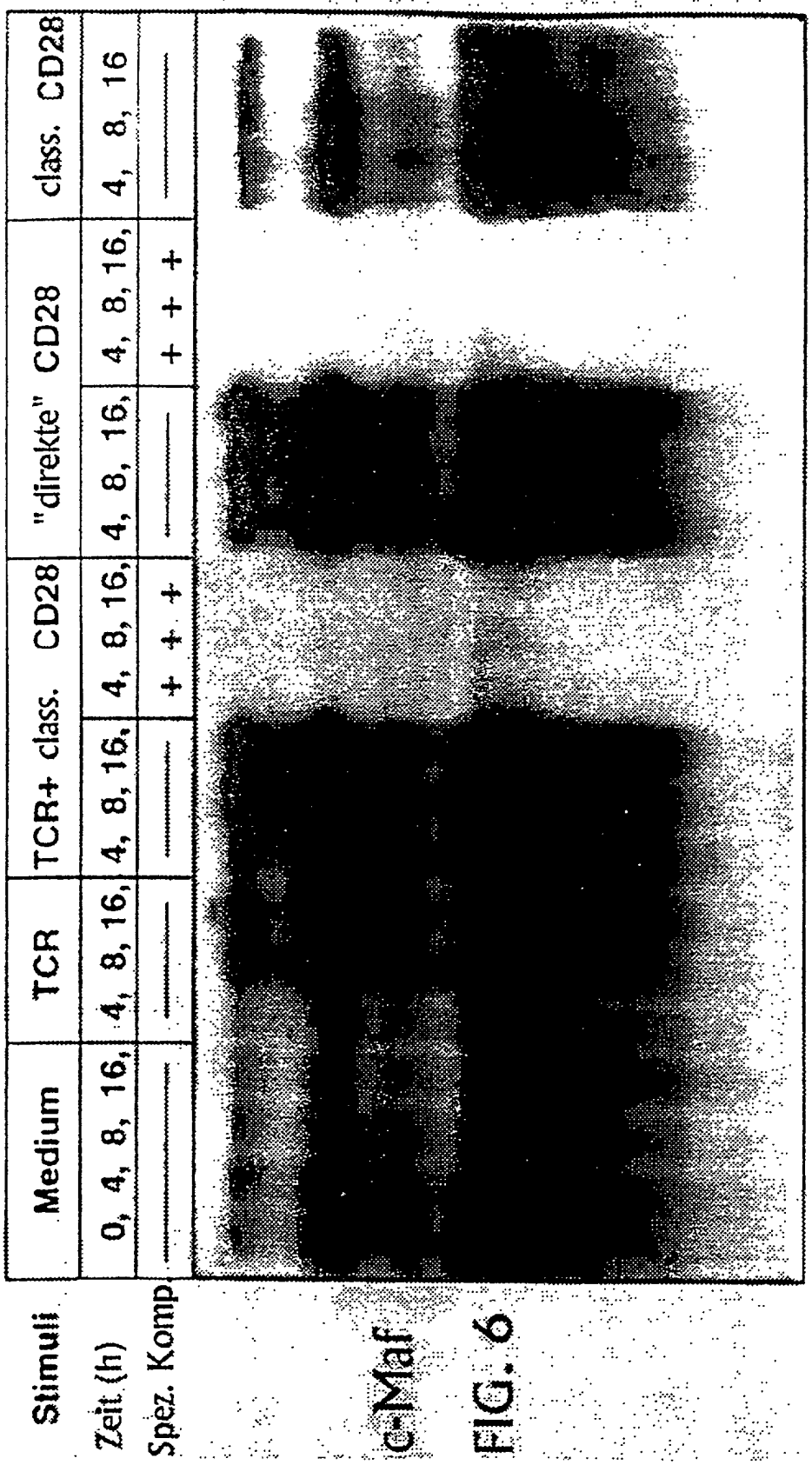
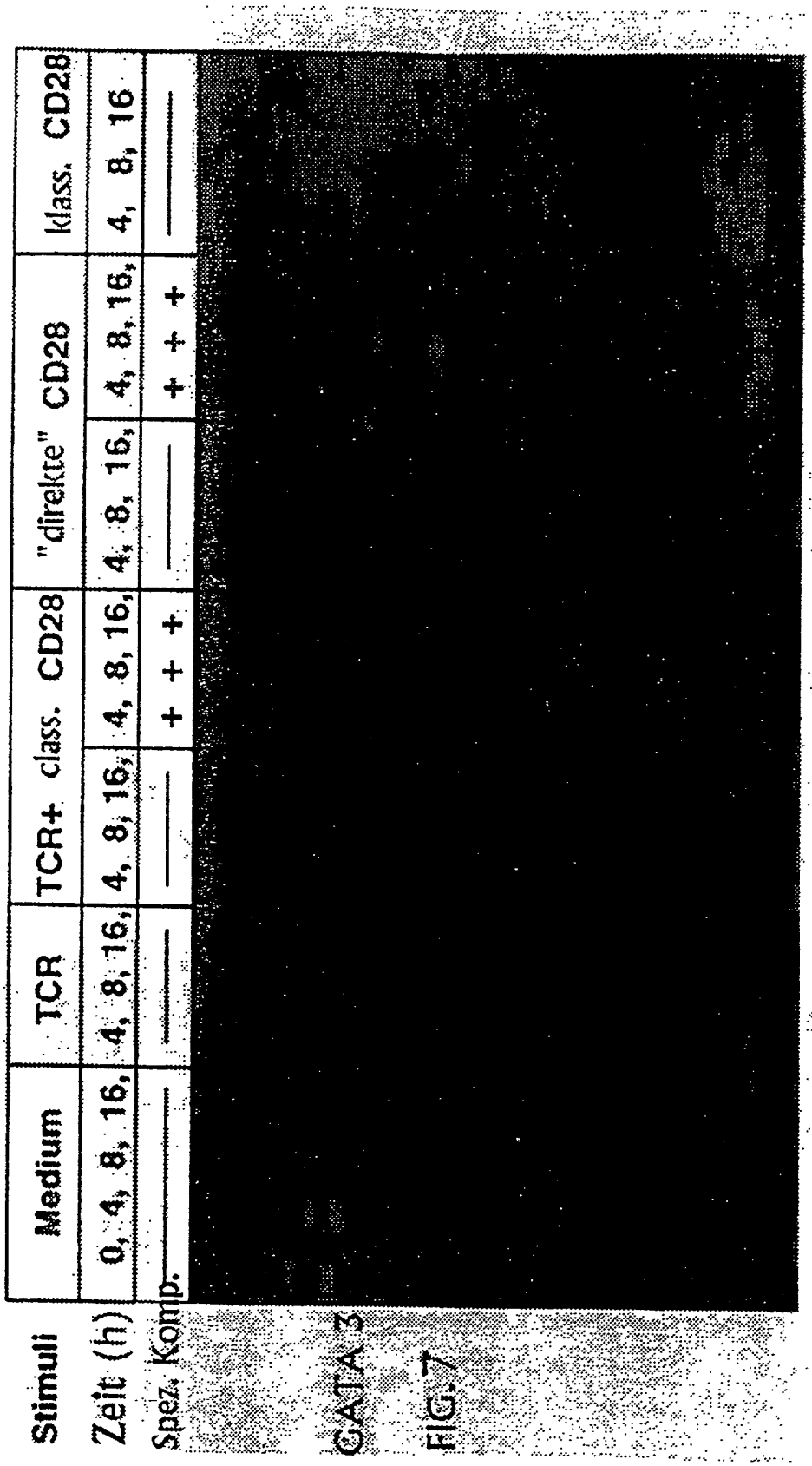

Stimuli
Zeit (h)
Spez. Komp.
Medium
0, 4, 8, 16,
TCR
4, 8, 16,
TCR+ class. CD28
4, 8, 16,
4, 8, 16,
"direkte" CD28
4, 8, 16,
4, 8, 16,
class. CD28
4, 8, 16
c-Maf

FIG. 6



Stimuli	Medium	TCR	TCR+ class. CD28			"direkte" CD28			Klass. CD28		
Zeit (h)	0, 4, 8, 16,	4, 8, 16,	4,	8,	16,	4,	8,	16,	4,	8,	16
Spez. Komp.	———	———	———	+	+	———	+	+	———	+	+

GATA 3

FIG. 7

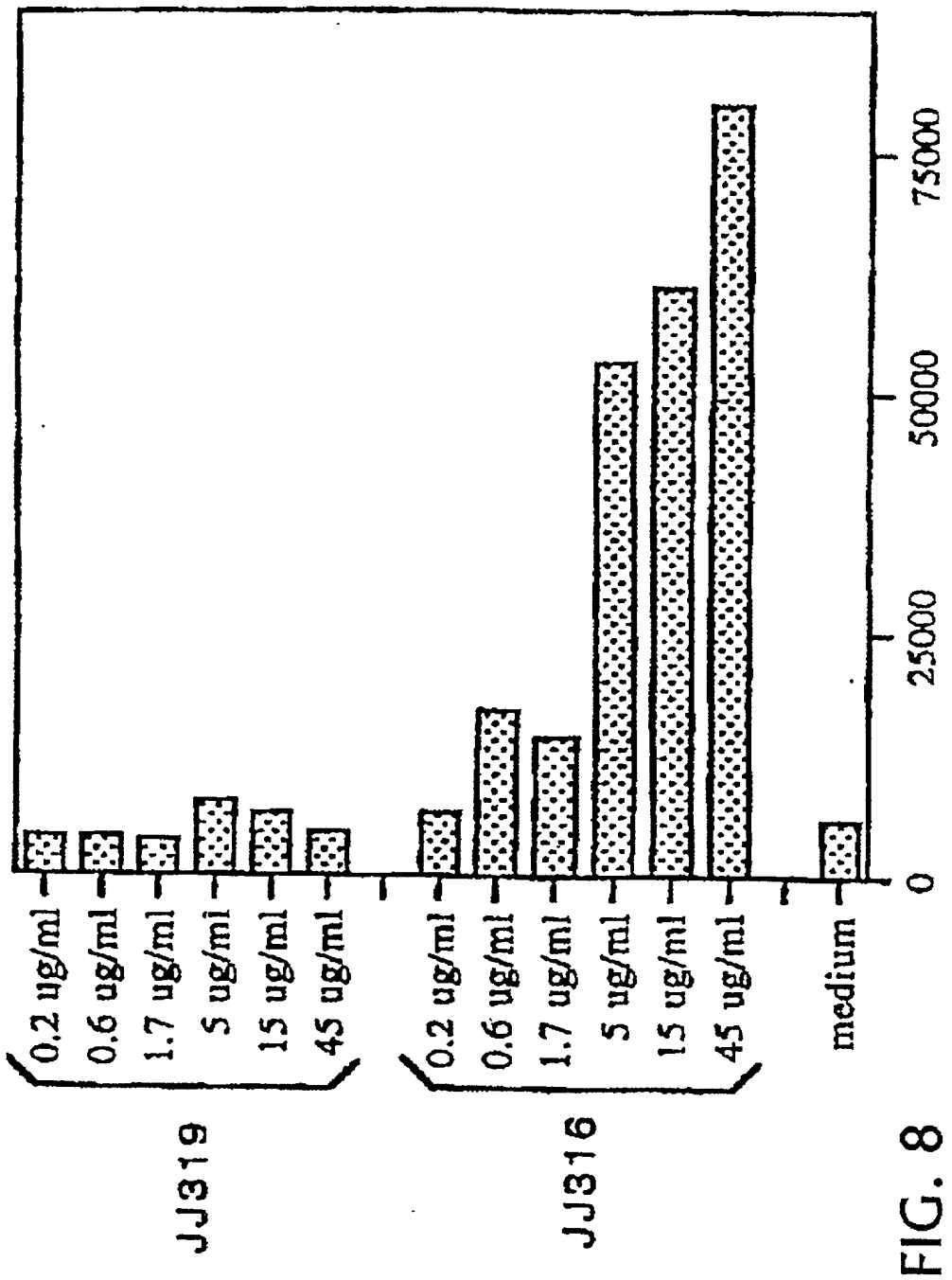


FIG. 8

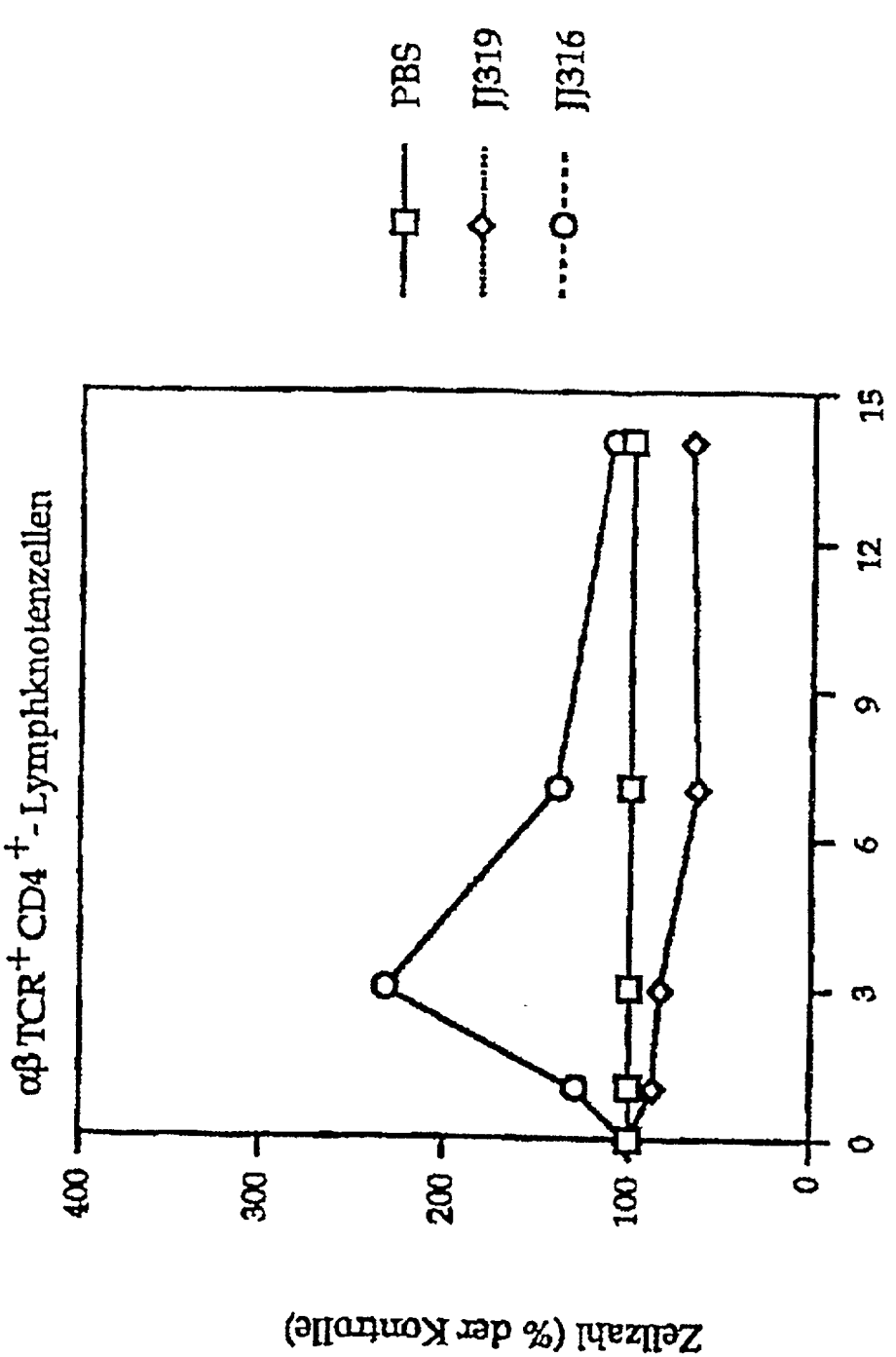

PBS
JJ319
JJ316
αβ TCR+ CD4+ - Lymphknotenzellen
400
300
200
100
0
0
3
6
9
12
15
Tage nach i.p. Injektion
Zellzahl (% der Kontrolle)

FIG. 9

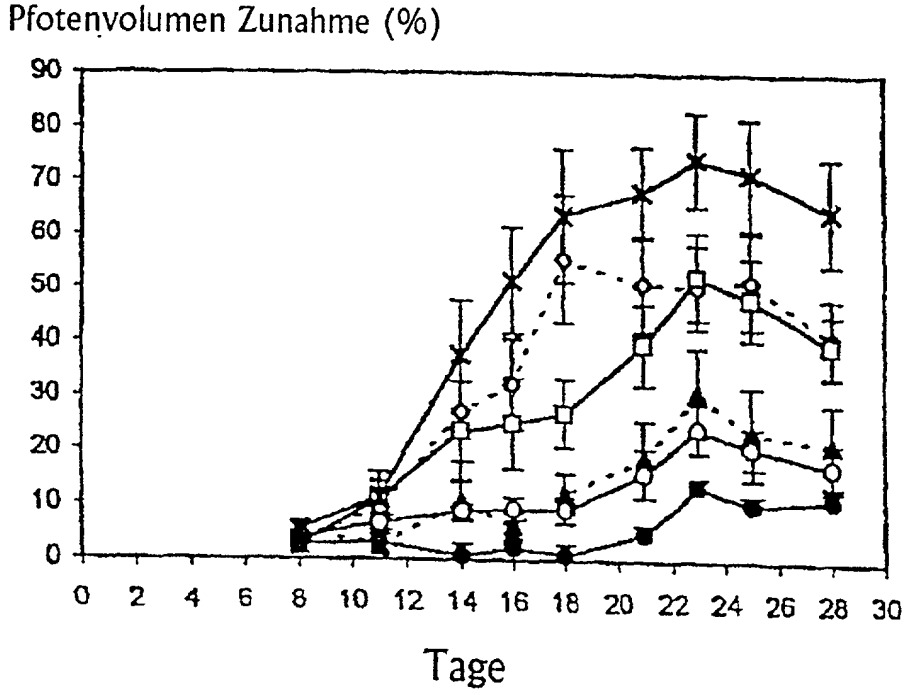
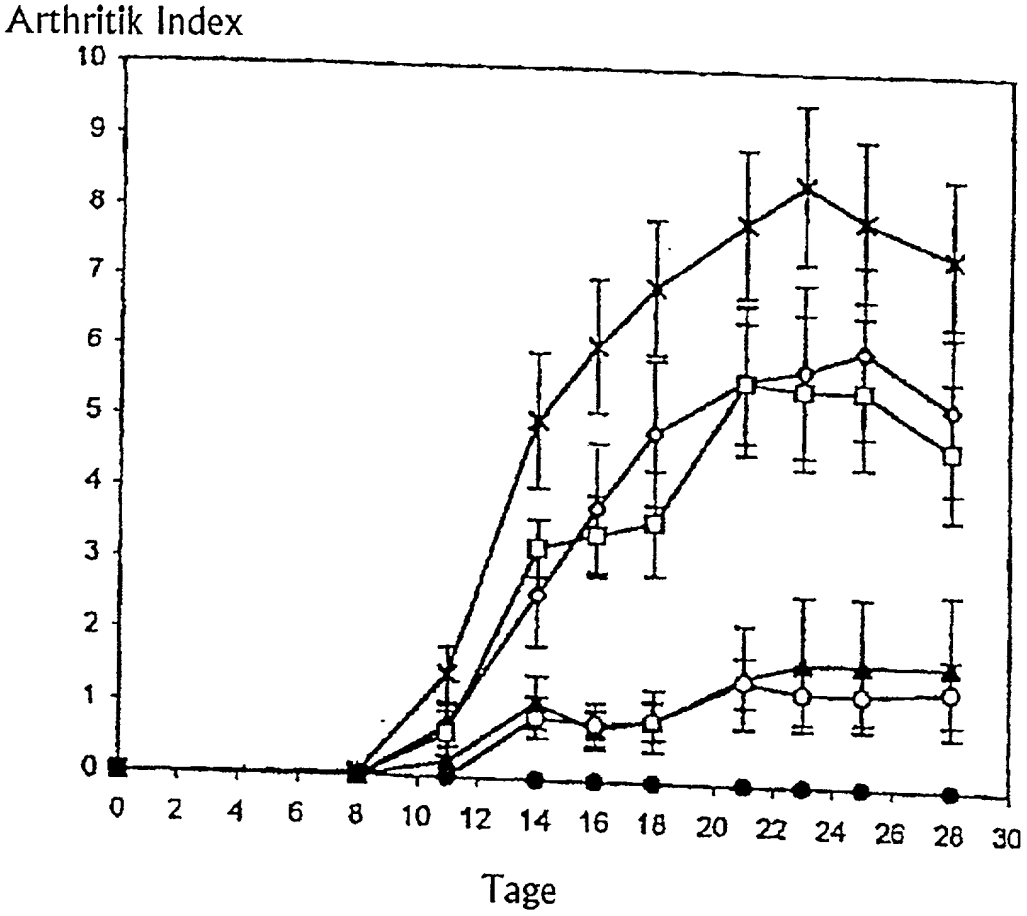


FIG. 10


Arthritik Index
10
9
8
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4
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0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Tage
AA + PBS
AA + Isotype control
AA + W3/25
AA + JJ316
AA + JJ319
gesund

FIG. 11